



PHD

In vivo ADP-ribosylation of proteins in mouse L1210 cells.

Surowy, Carol S.

Award date:
1981

Awarding institution:
University of Bath

[Link to publication](#)

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy

If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

IN VIVO ADP-RIBOSYLATION
OF PROTEINS IN
MOUSE L1210 CELLS

Submitted
by
CAROL S. SUROWY
for the degree of Ph.D.
of the
UNIVERSITY OF BATH
1981

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purpose of consultation.

Carol S. Surowy

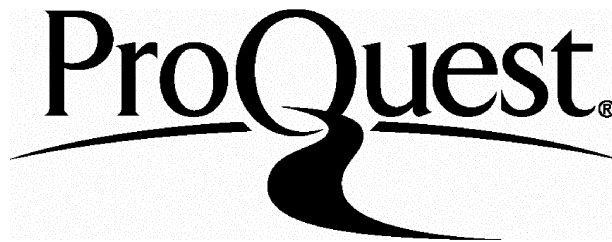
ProQuest Number: U311996

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest U311996

Published by ProQuest LLC(2015). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
LIBRARY

16 APR 1981

PhD

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. W.J.D. Whish, for his advice, encouragement and friendship throughout these studies and also Dr. P.R. Stone for his advice and support.

I am grateful to the Medical Research Council for financial support.

My thanks to Mrs. R. Surowy for her patience in typing this thesis.

ABSTRACT

A method was developed for the isolation of protein ADP-ribosylated in vivo, using mouse L1210 cells. The method exploited the differences in buoyant density of RNA/DNA and protein on caesium chloride density gradient centrifugation. In order that the method may be generally applicable, it was designed to be both non-selective and to ensure full recovery of ADP-ribosylated protein. Approximately 13% of the [³H]adenosine-labelled, acid-insoluble material banded at the top of the gradient and was assumed to be ADP-ribosylated protein.

The isolated ADP-ribosylated protein was characterized, using enzymic and chemical analyses, with respect to chain length, ADPR-protein linkage, and number and type of proteins modified. The presence of PR-AMP after snake venom phosphodiesterase digestion confirmed the existence of poly(ADP-ribosyl)ated protein in vivo. The characterization showed that the majority (approx. 85%) of material at the top of the gradient represented ADP-ribosylated protein. The average chain length of ADPR bound to protein was estimated as 1.3 ADPR units. 87% of material on top of the gradient was sensitive to base and 21% was sensitive to hydroxylamine. Of the material which remained acid-insoluble after treatment with base, approximately 55% represented base-resistant ADP-ribosylated protein. This suggests that there may be at least three types of linkage between ADPR and protein in vivo. Analysis of proteins by SDS gel electrophoresis revealed that a number of species were ADP-ribosylated.

To characterize the in vivo system further, the effects of 3-aminobenzamide and thymidine, which are known to inhibit poly(ADPR) synthetase in vitro, were studied. In contrast to results obtained in vitro, only partial inhibition of ADP-ribosylation was observed. Furthermore, the extent of inhibition appeared to decrease with time, being 65% at 6 hours but only 40% at 24 hours. The effect of the alkylating agent dimethyl sulphate on ADP-ribosylation in vivo was also studied. Addition of 105 μ M DMS resulted in a rapid but transitory decrease in NAD⁺ levels and a complementary increase in ADP-ribosylation of protein. This finding supports the hypothesis that ADP-ribosylation of proteins in vivo may be involved in DNA repair.

TO MY PARENTS

ABBREVIATIONS

3-AB	3-aminobenzamide
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
ADPR	Adenosine diphosphate ribose
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukemia
DABA	Diaminobenzoic acid
dADPR	Deoxyadenosine diphosphate ribose
dAMP	Deoxyadenosine monophosphate
dCTP	Deoxycytidine triphosphate
DMS	Dimethyl sulphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNAD ⁺	Deoxynicotinamide adenine dinucleotide
DNase	Deoxyribonuclease
dTMP	Deoxythymidine monophosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EAT	Ehrlich ascites tumour
EtOH	Ethanol
GMP	Guanosine monophosphate
HAc	Acetic Acid
HMBA	Hexamethylenebisacetamide
HMG	High mobility group
3-MB	3-methoxybenzamide

MNNG	N-methyl-N'-nitro-N-nitroguanidine
MNU	N-methyl-N-nitrosourea
M _r	Molecular ratio
MTT	Thiazolyl blue
NaAc	Sodium acetate
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NMN	Nicotinamide mononucleotide
PCA	Perchloric acid
PEI	Polyethyleneimine
PHA	Phytohaemagglutinin
PMS	Phenazine methosulphate
PMSF	Phenylmethanesulphonyl fluoride
PPO	2,5-diphenyloxazole
PR-AMP	2' (5"-phosphoribosyl)-5'AMP
R _f	Relative mobility
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
t.l.c.	Thin-layer chromatography
tRNA	Transfer ribonucleic acid
V _e	Elution volume
V _o	Void volume
XP	Xeroderma pigmentosum

TABLE OF CONTENTS

	<u>Page</u>
TITLE	(i)
ACKNOWLEDGEMENTS	(ii)
ABSTRACT	(iii)
DEDICATION	(v)
ABBREVIATIONS	(vi)
TABLE OF CONTENTS	(viii)

<u>CHAPTER 1</u>	<u>GENERAL INTRODUCTION</u>	1
1.1	INTRODUCTION	2
1.2	PROPERTIES AND STRUCTURAL CHARACTERISTICS OF POLY(ADPR)	5
1.3	SYNTHESIS OF POLY(ADPR)	10
1.4	DEGRADATION OF POLY(ADPR)	25
1.5	QUANTITATION	30
1.6	<u>IN VIVO</u> STUDIES	37
1.7	ADP-RIBOSYLATION OF NUCLEAR PROTEINS	47
1.8	BIOLOGICAL FUNCTION OF ADP-RIBOSYLATION	57
	1.8.1 DNA Replication	58
	1.8.2 Cell Proliferation	64
	1.8.3 DNA Transcription	67
	1.8.4 Cell Cycle	69
	1.8.5 Cell Differentiation and Development	72
	1.8.6 DNA Repair and Fragmentation	77
	1.8.7 ADP-Ribosylation and Chromatin Structure	84
1.9	CONCLUSION	92

	<u>Page</u>
<u>CHAPTER 2</u> <u>MATERIALS AND METHODS</u>	95
2.1 MATERIALS	96
2.1.1 General	96
2.1.2 Cell Culture	96
2.1.3 Radioactive Labels	96
2.1.4 Counting of Radioactive Samples	97
2.1.5 Chromatography	97
2.1.6 Caesium Chloride Density Gradient Centrifugation	98
2.1.7 Protein Estimation	98
2.1.8 DNA Estimation	98
2.1.9 NAD ⁺ Assay	98
2.1.10 Substrates and Reagents	98
2.1.11 Enzymes	99
2.1.12 Inhibitors	99
2.1.13 Cytotoxic Reagents	99
2.1.14 Gel Electrophoresis	100
2.2 METHODS	100
2.2.1 Cell Culture	100
2.2.2 Preparation of Samples for Counting by Trichloroacetic Acid Precipitation	102
2.2.3 Counting of Radioactive Samples	103
2.2.4 Isolation of Pig Thymus Nuclei	104
2.2.5 Isolation of Mouse L1210 Nuclei	105
2.2.6 [³ H]NAD ⁺ Incorporation into Isolated Nuclei	105
2.2.7 Biogel P10 Chromatography of [³ H]NAD ⁺ -Labelled Pig Thymus Nuclei	106
2.2.8 [³ H]Adenosine Incorporation by Mouse L1210 Cells	106

	<u>Page</u>
2.2.9 Harvesting of Cells Radioactively Labelled with [³ H]Adenosine	107
2.2.10 Caesium Chloride Density Gradient Centrifugation of [³ H]-Labelled Cells	107
2.2.11 Processing of Isolated [³ H]Adenosine- Labelled Material	108
2.2.12 Enzymic and Chemical Analyses of Isolated [³ H]Adenosine-Labelled Material	108
2.2.13 Ion-Exchange Thin-Layer Chromatography	109
2.2.14 Protein Estimation	112
2.2.15 DNA Estimation	113
2.2.16 NAD ⁺ Assay	114
2.2.17 Aminoethyl Cellulose Chromatography	115
2.2.18 SDS Gel Electrophoresis	116
 <u>CHAPTER 3</u> <u>A METHOD FOR THE ISOLATION OF</u> <u>PROTEIN ADP-RIBOSYLATED IN VIVO</u>	 120
3.1 INTRODUCTION	121
3.2 GROWTH CYCLE OF L1210 CELLS	124
3.3 PROCESSING OF MATERIAL FOR CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION	127
3.3.1 Charcoal Column	127
3.3.2 Biogel P10 Chromatography	128
3.3.3 Improved Techniques for Isolation of Acid-Insoluble Material	130
3.4 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION	132
3.5 DESALTING OF GRADIENT MATERIAL	135
3.5.1 Dialysis	135
3.5.2 TCA Precipitation	136

	<u>Page</u>
3.6 DETERMINATION OF ANY PROTEOLYTIC ACTIVITY DURING CsCl DENSITY GRADIENT CENTRIFUGATION	137
3.6.1 'Mixing' Experiment	137
3.6.2 Sephadex G-75 Chromatography	138
3.7 DISCUSSION	140
 <u>CHAPTER 4</u> <u>CHARACTERIZATION OF PROTEIN ADP-RIBOSYLATION IN VIVO</u>	 143
4.1 INTRODUCTION	144
4.2 ENZYMIC AND CHEMICAL HYDROLYSES OF ADP-RIBOSYLATED PROTEIN	146
4.2.1 Enzymic Hydrolysis	148
4.2.2 Chemical Hydrolysis	149
4.3 TLC ANALYSES OF THE PRODUCTS OF HYDROLYSIS	150
4.3.1 T.l.c. Analysis of Snake Venom Phosphodiesterase Digestion Before and After Treatment with Alkaline Phosphatase - Determination of Chain Length	150
4.3.2 T.l.c. Analysis of Base Digest	151
4.3.3 T.l.c. Analysis of Hydroxylamine Digest	154
4.3.4 T.l.c. Analysis of Crude Glycohydrolase Digest	155
4.3.5 T.l.c. Analysis of Deoxyribonuclease/ Snake Venom Phosphodiesterase/ Alkaline Phosphatase Digest	156
4.4 CAESIUM CHLORIDE DENSITY GRADIENT ANALYSIS OF BASE DIGEST	157
4.5 AMINOETHYL CELLULOSE COLUMN CHROMATOGRAPHY OF BASE DIGEST	158
4.6 DISTRIBUTION OF [³ H]ADENOSINE INCORPORATED INTO ACID-INSOLUBLE MATERIAL IN L1210 CELLS	160
4.7 TURNOVER OF ADP-RIBOSYLATED PROTEIN IN L1210 CELLS	161
4.8 ADP-RIBOSYLATION OF PROTEINS IN L1210 CELLS FROM STATIONARY PHASE OF GROWTH	162

	<u>Page</u>
4.9 : DETERMINATION OF [^{14}C]NICOTINAMIDE-LABELLED ACID-INSOLUBLE MATERIAL	163
4.10 SDS GEL ELECTROPHORESIS	164
4.11 DISCUSSION	166
 <u>CHAPTER 5</u> <u>THE EFFECT OF INHIBITORS OF</u> <u>POLY(ADPR) SYNTHETASE ON</u> <u>ADP-RIBOSYLATION IN VIVO</u>	 173
5.1 INTRODUCTION	174
5.2 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION	175
5.3 SNAKE VENOM PHOSPHODIESTERASE DIGESTION- CHAIN LENGTH ANALYSES	176
5.4 BASE HYDROLYSIS - ANALYSIS OF MONOMER/ POLYMER RATIO AND AMOUNT OF RNA PRESENT	177
5.5 AMINOETHYL CELLULOSE COLUMN CHROMATOGRAPHY	179
5.5.1 Chromatography	179
5.5.2 T.l.c. Analysis of 6M Acetic Acid Fraction	179
5.5.3 Snake Venom Phosphodiesterase Digestion of 1M Ethylamine Fraction	180
5.6 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF CELLS TREATED FOR 6 HOURS WITH INHIBITORS OF POLY(ADPR) SYNTHETASE	180
5.7 DISCUSSION	182
 <u>CHAPTER 6</u> <u>THE EFFECT OF DIMETHYL SULPHATE</u> <u>ON ADP-RIBOSYLATION IN VIVO</u>	 187
6.1 INTRODUCTION	188
6.2 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF L1210 CELLS TREATED WITH 105 μM DMS	190
6.3 NAD^+ ASSAY OF MOUSE L1210 CELLS TREATED WITH 105 μM DMS	192

	<u>Page</u>
6.4 DISCUSSION	193
<u>CHAPTER 7</u> <u>GENERAL DISCUSSION</u>	198
REFERENCES	204
PUBLICATIONS	221
APPENDIX	222

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

The biosynthesis of nicotinamide adenine dinucleotide (NAD^+) involves a step in which nicotinamide mononucleotide (NMN) and adenosine triphosphate (ATP) are coupled to form NAD^+ . The enzyme which catalyses this step, NAD pyrophosphorylase has been found to be located exclusively in the nucleus of the cell (Hogeboom & Schneider, 1952). This made it difficult to imagine that the function of NAD^+ was solely cytoplasmic i.e. as a coenzyme of the dehydrogenases; it seemed likely that NAD^+ played an additional role in the eukaryotic cell. In fact, as early as 1958 Morton (1958, 1961) suggested that the nucleus may be sensitive to changes in NAD^+ concentration and hence that NAD^+ may play a significant role in the control of cellular proliferation. The importance of NAD^+ in nuclear function is further supported by more recent results from Rechsteiner et al. (1976) who showed, by a double label pulse chase method, that the half-life of NAD^+ in nucleated cells was approximately 1 hour i.e. it exhibits a surprisingly high turnover. Rechsteiner (Rechsteiner & Catanzarite, 1974) had previously shown that the half-life of NAD^+ in cells enucleated by cytochalasin was 10 hours. In addition it was shown (Hillyard et al., 1973) that the turnover of NAD^+ in the nucleus involves up to 95% of the total cellular NAD^+ content. The picture which emerges is that the nucleus seems to be the principal site of NAD^+ synthesis and breakdown and, moreover, NAD^+ may play a role in control of nuclear function.

Analysis of the NAD^+ molecule shows it to contain two sites at which the molecule is capable of acting as a substrate in transfer reactions, in addition to its role as a coenzyme, i.e. the pyrophosphate bond and the glycosidic linkage at the quaternary nitrogen of the pyridine ring (Zatman et al., 1953).

In 1962 work by Revel & Mandel demonstrated the existence of an enzymic system which catalysed the incorporation of $[^{14}\text{C}]\text{ATP}$ into an acid-insoluble product. This incorporation was stimulated 1,000-fold on addition of NMN (Chambon et al., 1963). It was shown that the adenylate moiety of ATP and the ribose and phosphate of NMN were incorporated. In addition, NAD^+ was found to be a better precursor than NMN and ATP and is, in fact, the immediate precursor of the reaction (Chambon et al., 1966; Nishizuka et al., 1967; Sugimura et al., 1967). Preliminary analysis of the acid-insoluble material was made by digestion with snake venom phosphodiesterase. The product of this digestion was a nucleotide with a ratio of adenine:ribose:phosphate of 1:2:2 (Chambon et al., 1966). This suggested that the acid-insoluble material was a polymer of adenosine diphosphate ribose (ADPR). The structure of the polymer, in particular the nature of the ribose-ribose linkage (between the C_2 of one ribose and the C_1 of the other), was first elucidated by Doly & Petek (1966). The formation of poly(ADPR) therefore seemed to occur by a transglycosylation of the ADPR moiety of NAD^+ with the simultaneous removal of nicotinamide. The energy for the reaction (34kJ/mol) is produced on

cleavage of the nicotinamide-ribose bond. The discovery of this reaction pathway provided not only an explanation for the high turnover of NAD^+ in the nucleus but also perhaps, because of the unique structure of the product, an insight into its possible significance.

In 1968 the significance of ADP-ribosylation in nuclear function was further substantiated by the discovery of poly(ADPR) covalently bound to nuclear proteins (Nishizuka et al., 1968). The acid-insoluble material was found to be associated with various protein fractions, in particular the histones. That the linkage of poly(ADPR) to proteins is covalent was demonstrated by the experiments of Otake et al. (1969) who performed caesium chloride density gradient centrifugation in the presence of guanidinium chloride; protein and poly(ADPR) bands coincided.

ADP-ribosylation of nuclear proteins represents another class of post-translational modification of nuclear proteins in addition to methylation (Paik & Kim, 1971), phosphorylation (Rubin & Rosen, 1975; Kleinsmith, 1978) and acetylation (Delange & Smith, 1971). These modifications are believed to be involved in some of the finer aspects of regulation of nuclear function. ADP-ribosylation is unique, however, in that the chain length may vary from 1 to as great as 65. In this way its regulatory capabilities may be even more subtle than those of the other known post-synthetic modifications.

Within the preceding pages a brief introduction to the discovery and significance of poly(ADPR) has

been given. Although a large amount of research has been carried out since its discovery the function of poly(ADPR) and ADP-ribosylation of nuclear proteins is still not understood. Thus, in the following pages it is intended to review the work to date with particular emphasis on the advantages and disadvantages of the methodology used in the field in an attempt to highlight some of the problems encountered during the study of poly(ADPR). In this way it is hoped to reveal the approaches that may best increase our understanding of the system in future.

In addition, since this review will only be concerned with ADP-ribosylation in the eukaryotic nucleus, it must be mentioned that bacterial and other ADP-ribosylation systems also exist (see Hayaishi & Ueda, 1977).

1.2 PROPERTIES AND STRUCTURAL CHARACTERISTICS OF POLY(ADPR)

As a polynucleotide poly(ADPR) displays the general characteristics of this class of macromolecule, being soluble in neutral and alkaline solutions but precipitated by 5% (w/v) trichloroacetic acid (TCA) or 66% (v/v) EtOH (Fujimura & Sugimura, 1971). Its precipitation by TCA or EtOH is routinely used during isolation of the polymer. The purified product has been analysed both by its absorption spectrum and its behaviour on density gradients. Purified poly(ADPR) exhibits an absorption maximum of 258nm at pH 7 (Reeder et al., 1967) and has an $A_{280}:A_{260}$ ratio of 0.26 (Shima et al., 1970). On sucrose gradients

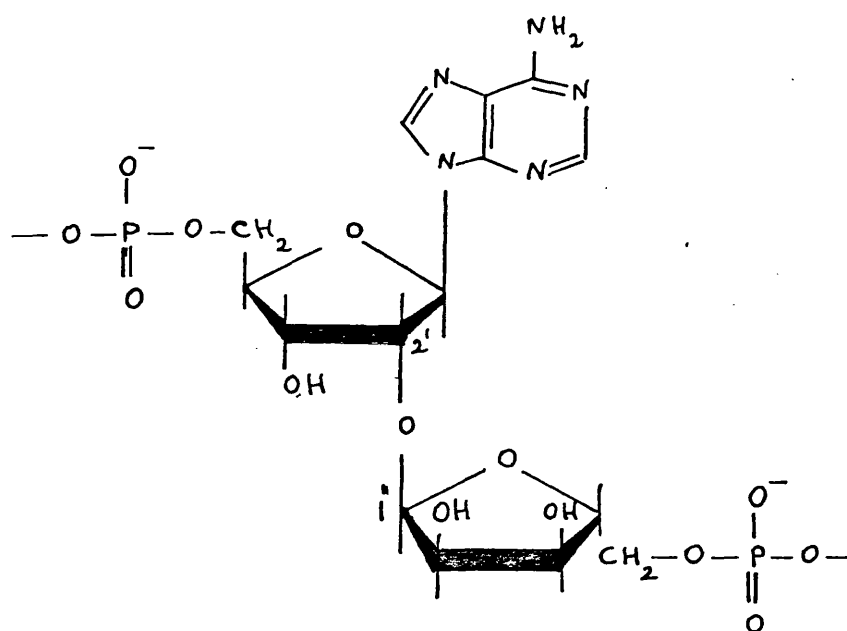
the polymer peaks at a value of 4S (Chambon et al., 1966; Hasegawa et al., 1967; Reeder et al., 1967) and on caesium sulphate equilibrium density gradient centrifugation it exhibits a buoyant density of 1.57, just lighter than transfer RNA (Hasegawa et al., 1967). As an O-glycoside the polymer is stable in alkali, even when incubated in 2M NaOH at 37°C for 18 hours (Hasegawa et al., 1967; Nishizuka et al., 1967). However, on incubation in alkali, 75% of free ADPR is converted to 5'AMP (Goebel et al., 1977). Under these conditions an unknown compound 'X' may also be found. This difference in susceptibility to alkali has been used to selectively analyse mono and poly(ADPR) (Goebel et al., 1972; Purnell et al., (1980b)). The polymer is stable in cold acid but if boiled in 1M HCl for 7 minutes is cleaved at the ribose-ribose bond (Hasegawa et al., 1967). Enzymic digestion of poly(ADPR), which was used in a further attempt to elucidate its structure, revealed that snake venom phosphodiesterase was the only enzyme capable of cleaving the polymer, the products being 2' (5"-phosphoribosyl)-5'AMP and 5'AMP (Nishizuka et al., 1967). This insensitivity towards other enzymes has proved useful in attempts to isolate the polymer from other macromolecules (Shima et al., 1970; Sugimura et al., 1971).

Since the preliminary work of Doly (Chambon et al., 1966) who elucidated the structure of poly(ADPR) by chemical means, subsequent n.m.r. analyses using both pure poly(ADPR) (Miwa et al., 1977) and 2'-(5"-phosphoribosyl)-5'AMP, its degradation product (Miwa et al., 1977;

Ferro & Oppenheimer, 1978; Inagaki et al., 1978), have substantiated that the linkage between one ADPR unit and the next is α -(1"-2'). By using n.m.r. the configuration about the anomeric carbon was determined. See Fig. 1.2. In addition, Suhadolnik et al. (1977) have shown, by the use of the NAD⁺ analogue 2'dNAD⁺, that a 1"-3' osidic linkage is possible. The significance of this finding, however, is not yet known.

The analysis and identification of poly(ADPR) has been greatly facilitated by the use of snake venom phosphodiesterase. The enzyme cleaves the polymer endonucleolytically to give the unique product phosphoribosyl-AMP from the internal residues and 5'AMP from the terminal ADPR residue. The chain length of poly(ADPR) was first determined by Nishizuka et al. (1969) using this enzyme. The products were separated by paper chromatography and the ratio of total radioactivity (PR-AMP plus 5'AMP) to that in 5'AMP gave an average chain length value. Since the 5'AMP only arises from the terminal residue the number of chains could also be calculated. Analysis of poly(ADPR) in this way, using a variety of different tissues and species, revealed that the chain length of the polymer varied substantially. Since the chain length obtained by this method is only an average, it was realized that the range of values within this might be substantial. In fact, Adamietz & Hilz (1976) showed that on caesium sulphate density gradient centrifugation poly(ADPR) from a variety of tissues exhibited a spectrum of different sizes.

Fig. 1.2 Structure of the Ribosyl(1" - 2')Ribose
Glycosidic Linkage in Poly(ADPR)



The first attempt at resolving polymer of differing sizes was that of Sugimura et al., (1971). [^{14}C] NAD^+ -labelled nuclei were freed from protein by digestion with Pronase and the remaining DNA, RNA and poly(ADPR) subjected to hydroxyapatite chromatography under increasing concentration of phosphate buffer. A direct relationship between chain length and phosphate concentration as well as a good separation of poly(ADPR) from DNA and RNA was observed. By submitting the oligo(ADPR) peaks to electrophoresis it was found that each fraction was comprised of two subfractions, differing in both chain length and terminal structure (Tanaka et al., 1977). Adamietz et al. (1978) used the high resolution obtained with polyacrylamide gel electrophoresis and the susceptibility of the protein-poly(ADPR) linkage to alkali (Nishizuka et al., 1969) to analyse individual polymer chains obtained from [^3H] NAD^+ -labelled nuclei. Chain lengths of at least 33 units were resolved, the size distribution being visualized by fluorography (because of its excellent resolution) and quantitated by counting the radioactive content of each gel slice. Using this method Adamietz was able to demonstrate quantitative differences in the chain length pattern of nuclei from different tissues. Tanaka et al. (1978) modified their earlier method of fractionating poly(ADPR), using a combination of hydroxyapatite chromatography and gel electrophoresis, to demonstrate the presence of at least 65 discrete species of polymer. Analysis of the chain length of each band by the conventional method of Nishizuka et al. (1969) showed

chain lengths of up to (ADPR)₃₀, but no greater. They suggested that this may be due to the presence of a branched structure with two or more AMP termini. The existence of much larger poly(ADPR) on gels was also demonstrated. The work was extended (Miwa et al., 1979) by analysing the hydrolysis products of a snake venom phosphodiesterase digest on the high molecular weight polymer fraction obtained from hydroxyapatite chromatography. By using a combination of thin-layer chromatographic and mass spectrometric techniques the existence of a new compound, 2' [1"-ribosyl-2" (or 3")-1"ribosyl] adenosine-5',5",5""-tris (phosphate) which constituted 2% of the reaction products, was demonstrated. This finding provided evidence in support of a branched structure whereby additional ADPR molecules are added, via a 1"-2" or 1"-3" glycosidic linkage, to the ribose on which nicotinamide was originally attached. This introduced new concepts into the structure and function of poly(ADPR) as well as the enzymology of the ADPR system. Farzaneh & Pearson (1978) also used hydroxyapatite to determine the chain lengths of poly(ADPR) during the development of Xenopus laevis. By comparing the values obtained with those of the phosphodiesterase method of Nishizuka et al. (1969), Farzaneh & Pearson found that the hydroxyapatite method consistently gave larger values. They proposed that [³H]NAD⁺ was incorporated in vitro on to pre-existing chains which were synthesized in vivo. The significance of this finding with respect to any chain length studies in vitro, including those described above,

is easily seen. In addition, it raises the question of whether poly(ADPR) synthetase is capable of initiation as well as elongation (see Section 1.3), but in particular it demonstrates the importance of in vivo work (see Section 1.6). Average chain length determinations will also be influenced by any endogenous degradative enzymes such as poly(ADPR) glycohydrolase and phosphodiesterase, and particular caution should be taken during isolation of poly(ADPR) under conditions which may activate these enzymes.

1.3 SYNTHESIS OF POLY(ADPR)

The enzyme which catalyses the formation of poly(ADPR) from NAD^+ is an ADP-ribosyl transferase called poly(ADPR) polymerase or poly(ADPR) synthetase.

One approach to characterizing the formation of poly(ADPR) has been to purify the synthetase itself. The first attempts at purification basically involved isolating chromatin and then purifying the synthetase from DNA and other macromolecules by a combination of chromatographic techniques and ammonium sulphate precipitation (Yoshihara, 1972; Ueda et al., 1975; Tsopanakis et al., 1976; Okayama et al., 1977). Okayama et al. found that the enzyme was very unstable after its separation from DNA. Khan & Shall (1976) were the first to attempt a specific purification of the synthetase by employing affinity chromatography. By coupling nicotinamide, an inhibitor of the synthetase, or Dextran blue, which has a chromophore structure similar to NAD^+ , to Sepharose, they were able to achieve an 85-fold and 34-fold purification of the synthetase, respectively. In

both cases a greater than 100% yield was obtained which suggested the removal of an endogenous inhibitor or degradative enzyme(s) during the purification step. The affinity of the synthetase for nicotinamide and Dextran blue was also used, in combination with other chromatographic techniques, by Mandel's group (Okazaki et al., 1976; Mandel et al., 1977) and Hotlund et al. (1980) who suggested (Kristensen & Hotlund, 1980) that the dye may interact with the synthetase at a site which normally binds nucleic acids. These studies yielded greater fold purification but lower percentage yield than those of Khan & Shall (1976).

The affinity of poly(ADPR) synthetase for DNA was first exploited by Kristensen & Hotlund (1976) in an attempt to purify the enzyme. By employing a two-step procedure of phosphate extraction and DNA-agarose chromatography they were able to purify the synthetase to high specific activity. By including an isoelectric focusing step the purification was increased even further (Kristensen & Hotlund, 1978). Yoshihara et al. (1978) and Ito et al. (1979) also used DNA affinity steps and were able to purify the synthetase to apparent homogeneity. Tsopanakis et al. (1978) realized that the extreme lability and therefore inactivation of the synthetase during isolation was a major obstacle in the successful purification of the enzyme. In an attempt to overcome this problem they employed an organic solvent (50% (v/v) ethylene glycol) at sub-zero (-10°C) temperatures to achieve a 9,200-fold purification with 46% yield.

The molecular weight of purified synthetase has been determined using sedimentation equilibrium, gel filtration and gel electrophoresis and ranges from 63,000 in pig thymus (Tsopanakis et al., 1978) to 130,000 in calf thymus (Yoshihara et al., 1978) with good agreement between the methods used. Although the synthetase appears to be a single polypeptide chain and can at least elongate poly-(ADPR) chains in both cases, it is not known whether the different values reflect real differences in the structure of the enzymes or arise during the different purification steps involved. Sedimentation data (Ito et al., 1979) suggest that the enzyme is a globular protein with slight asymmetry. The pI value, as determined by isoelectric focusing, varies from 9.8 in calf thymus (Ito et al., 1979) to 8.4 in pig thymus (Tsopanakis et al., 1978). N-terminal (Ito et al., 1979; Tsopanakis et al., 1978) and amino acid (Ito et al., 1979; Kristensen & Hotlund, 1980) analyses showed that the N-terminus appeared to be blocked and that the synthetase contained a high proportion of residues that are either acidic or basic. This latter feature has been reported for other chromatin proteins, including the high mobility group proteins (Goodwin & Johns, 1977) and a non-histone protein from hen oviduct (Teng et al., 1978). Whether this high proportion of charged residues is linked with some particular structural or functional feature of these proteins is not clear, but may become evident on further characterization of other chromatin proteins.

Like NAD pyrophosphorylase, poly(ADPR) synthetase was found to be located exclusively in the nucleus of the cell. By a combination of cellular and nuclear fractionation

techniques it was shown that approximately 90% of the activity was associated with chromatin (Nishizuka et al., 1969) and that this activity was tightly bound to DNA (Ueda et al., 1968). Hilz & Kittler (1968) also demonstrated, by autoradiography, that poly(ADPR) synthetase was present throughout nuclei, there being little activity in the nucleoli. More recent results (Furneaux & Pearson, 1980) have confirmed these original findings.

Analysis of the properties of the synthetase reaction in isolated nuclei and more recently with purified or partially purified enzyme have shown it to be stimulated, but not dependent on, Mg^{2+} and a thiol reagent. The stimulation by Mg^{2+} in LS cells was found to be dependent on the NAD^+ concentration used (Stone & Shall, 1973). Its stimulation by thiol reagents suggests that the enzyme contains SH groups and requires these in a reduced form for maximal activity. In addition, polar compounds, including $(NH_4)_2SO_4$ (Whitby, 1980) have been found to inhibit the synthetase activity whereas non-polar compounds stimulate it (Kristensen & Hotlund, 1978). It was suggested that this may indicate that an ionic bond is necessary at some stage of the catalytic process. The pH optimum of the enzyme is approximately 8 and the temperature optimum is usually some 10-15°C lower than the optimum growing temperature of the cells or tissue from which the enzyme is isolated. This latter phenomenon has been difficult to explain in terms of the function of poly(ADPR). Gill (1972) showed that it was not a result

of differences in initial rate of formation of poly(ADPR) or rate of denaturation of the enzyme and suggested that the effect was brought about by some means other than directly on the enzyme, possibly through changes in configuration of the polymer itself. Alternatively, it may be due to a decreased rate of degradation of the polymer at reduced temperatures. It may be that the observed high activity of the synthetase at low temperatures is related to cellular events which bring about a release of 'latent' activity as described in 1.8.6.

The affinity of the enzyme for NAD^+ seems to differ between tissues, and may vary at different stages of the cell cycle (Brightwell et al., 1975). K_m values obtained have varied from $23\mu\text{M}$ in pig thymus (Tsopanakis et al., 1978) to 1.5mM in LS cells (Stone & Shall, 1973). However, such values have been estimated under a variety of different conditions and purification states. In fact, in the presence of histones the K_m and V_{max} were decreased and increased, respectively (Okayama et al., 1977) and in the presence of DNA the K_m was found to vary considerably with DNA concentration (Niedergang et al., 1979). The conditions in which these kinetic parameters are measured may therefore contribute significantly to the difference in values obtained. In addition, Furneaux & Pearson (1980) obtained an enzyme preparation from BHK-21/C13 cells which displayed biphasic Michaelis-Menten kinetics. Although the presence of endogenous activators or inhibitors may have contributed to this phenomenon, it is nevertheless an interesting observation in relation to the function of poly(ADPR)

(see below).

The specificity of the synthetase for β -NAD⁺ is high, neither NADP⁺ nor NADH being substrates. More recently, however, Suhadolnik et al. (1977) have shown that various NAD⁺ analogues altered in the AMP moiety were able to incorporate ADPR, but at slower rates than NAD⁺.

In addition to binding tightly to DNA, studies on the purified synthetase have shown that it has an absolute requirement for double-stranded DNA (Yoshihara, 1972; Yoshihara et al., 1978; Ito et al., 1979). The presence of DNA brings about both chain elongation (Nishizuka et al., 1969) and an increase in chain number (Yamada & Sugimura, 1973). Enzyme preparations not purified to homogeneity did not possess this absolute requirement probably due to contaminating DNA, as suggested by Mandel et al. (1977). Using purified synthetase from calf thymus Yoshihara et al. (1978) compared, by enzyme saturation studies, the ability of various DNA species to support poly(ADPR) synthetase activity. It was shown that the concentration of DNA required for half maximal enzyme activity was proportional to the enzyme concentration in the reaction mixture and, furthermore, that the number of base pairs to achieve this was approximately 200 with bulk calf thymus DNA but only 10 with an 'active' DNA fraction recovered during purification. This 'active' DNA has been isolated and partially characterized; it had a GC content of 43% (Hashida et al., 1979). These results suggested that the enzyme is activated by binding to a specific site on the DNA. Neidergang et al. (1979)

reached much the same conclusion by making a comparison of purified DNA-dependent and DNA-independent enzymes. On removal of the specific fraction of DNA in association with the DNA-independent enzyme by hydroxyapatite chromatography the enzyme became DNA-dependent. The activity of this enzyme could be restored by adding bulk calf thymus DNA or the specific fraction of DNA from which it was originally freed. However, the concentration of calf thymus DNA needed was a hundred times higher than that of the specific DNA fragment. The minimum number of base pairs of this specific DNA required for maximal enzyme activity was 16. By comparing the physicochemical and kinetic properties of the two enzymes Niedergang showed that the DNA-independent enzyme was better adapted to supporting enzymic activity of poly(ADPR) synthetase. This study brings into question the importance of purifying an enzyme to absolute homogeneity to study its activity; the conditions under which the enzyme is being studied may deviate widely from those which exist in vivo. Kristensen & Hotlund (1978) proposed, on the basis of preincubation studies, that the stimulatory effect of DNA is due to a reduction in the inhibitory effect of poly(ADPR) on its own synthesis, the two polymers being in competition for the same site on the enzyme.

Cleavage of DNA by DNase has been shown to either inhibit or stimulate the activity of the synthetase (Sugimura et al., 1973; Janakedevi & Koh, 1974). Janakedevi & Koh showed that stimulation occurred at low DNase concentrations but inhibition was seen at high levels of the enzyme. Gill et al. (1974) demonstrated that

sheared DNA enhanced synthetase activity more than unsheared DNA, suggesting that the synthetase may recognise ends of DNA molecules as opposed to a particular base sequence. It may be that the 'active' DNA described above was a consequence of the end of the molecule rather than a particular sequence. Miller (1975a,b), Roberts et al. (1975) and Benjamin & Gill (1978) also demonstrated an activation effect by fragmented DNA. Thus it seems that fragmented DNA stimulates poly(ADPR) synthetase and it may be that extensive fragmentation by, for example, DNase is inhibitory because DNA in this state is no longer able to support the enzyme. Recently, Yoshihara et al. (1980) have shown that calf thymus DNA treated with various DNases increases the enzyme activating efficiency to almost the same level as 'active' DNA. Whether the stimulation of the synthetase by 'active' DNA or fragmented DNA is related, is at present unknown.

Histones have been reported to stimulate the activity of purified synthetase 2-3-fold in the presence of DNA (e.g. Okayama et al., 1977). The stimulation was found to increase the chain length of poly(ADPR) but not the number of chains synthesized (Okayama et al., 1977; Ito et al., 1979). Ito et al. (1979) proposed that the stimulation by histone was by allosteric activation, whereas Yoshihara et al. (1978) showed that it may be due to masking of the inhibitory effect of denatured DNA. In fact, the latter group showed that although histones stimulated ADP-ribosylation by 100 or 400% with intact or partially denatured DNA, respectively, the activity was still near maximal even without histones in the presence of 'active' DNA

or poly(dA)•poly(dT); the stimulation as such was therefore dependent on the type of DNA present. This finding does not, however, preclude some role for histone in the stimulation of synthetase activity. The concentration of histone needed for stimulation was in fact found to be dependent on the concentration of DNA. Tsopanakis et al. (1978), in contrast to previous reports, showed an absolute requirement of purified pig thymus synthetase for histone. However, in analysing the response to histone, high molecular weight DNA which produces a greater stimulatory effect by histone than native DNA, was added to the assay. Therefore, whether the effect they observed is real or a result of such great stimulation as to appear histone-dependent remains to be seen. Further work on the effect of histone on synthetase activity by Tanaka et al. (1978) showed that when Mg^{2+} was omitted from the reaction mixture purified synthetase activity became almost completely dependent on histone. Chain length analyses showed that the length of polymer synthesized was shorter in the histone-dependent reaction compared to the Mg^{2+} -dependent reaction. Using histone H1, which of all histones was best able to stimulate synthetase, Tanaka et al. showed that in the histone-dependent reaction histone H1 acted as an acceptor of poly(ADPR). However, on increasing the DNA:histone H1 ratio the histone dependent reaction disappeared. They interpreted these results to mean that H1 bound to DNA cannot act as an acceptor; only when the H1:DNA ratio is high may such a reaction occur. These studies prove interesting with respect to local concentration effects of Mg^{2+} , histone and DNA relative to each

other, on the chromatin, and their relationship to poly(ADPR). Thus, the formation of protein-bound poly(ADPR) may only occur under a particular set of conditions.

One aspect of the poly(ADPR) system which still remains in question is whether the synthetase is capable of chain initiation as well as chain elongation, or whether a separate 'initiating' enzyme carries out this step. In vitro chain lengths analysed under conditions optimal for synthetase activity may only reflect the elongating capability of the enzyme as suggested by the results of Farzaneh & Pearson (1978). The availability of purified synthetase has thus proved useful in an attempt to solve this problem. Although the synthetase was capable of synthesizing poly(ADPR) and of elongating an ADPR-histone H1 conjugate (Ueda et al., 1979), it was the experiments of Kawaichi et al. (1980) using the same purified enzyme preparation that showed elongating capabilities, which demonstrated the initiation of poly(ADP-ribosyl)histone H1. The initiation was brought about by increasing the histone concentration compared to that previously used. However, whether such an increase in local concentration may bring about the same effect in vivo i.e. whether such a mechanism occurs naturally, is unknown. The initiating capability was demonstrated by isolation of labelled ribose-phosphate after alkaline hydrolysis and snake venom phosphodiesterase digestion of the reaction product. In addition, the protein-ADPR bond was unstable when treated with NaOH or NH₂OH; the results suggested that initiation was similar, in terms of linkage

and acceptor proteins, to that previously described. Thus, it seemed that the purified synthetase was capable of catalysing two different reactions, the formation of a glycosidic and an ester bond and, as a result, catalyses both chain elongation and chain initiation.

However, the observation of two different K_m values for NAD^+ in nuclei from S phase versus G2 phase (Kidwell & Burdette, 1974) and for formation of short ADPR chains versus poly(ADPR) chains (Dietrich & Siebert, 1973), in addition to the biphasic Michaelis-Menten kinetics observed by Furneaux & Pearson (1980) in BHK-21/Cl3 cells, suggests the existence of more than one enzyme catalysing the transfer of ADPR residues from NAD^+ to certain acceptors. The existence of at least two different types of linkage between ADPR and nuclear proteins (Smith & Stocken, 1975; Adamietz & Hilz, 1976) also supports this theory.

In 1977, Yoshihara et al. reported that the purified synthetase was capable of initiating self-ADP-ribosylation in the absence of any exogenous acceptor protein. Analysis by gel filtration and SDS polyacrylamide gel electrophoresis showed the enzyme and radioactivity to coincide as one peak. In addition, the bound radioactivity was shown to be oligo(ADPR) with an average chain length of 2.6. The linkage of the enzyme-bound early product was suggested to be of a covalent nature by its stability in SDS and urea. By lengthening the incubation time it was found that the polymer and enzyme became separated on SDS polyacrylamide gel electrophoresis; it may be that

once it has reached a certain length the polymer chain is released from the enzyme, so allowing the initiation of a new chain. Specificity with regard to polymer chain length may be determined by the acceptor protein itself; the chain may be released and transferred to the acceptor protein after varying lengths of time according to the structure of that protein. Kristensen & Hotlund (1976, 1978) have also suggested that the synthetase may serve as an acceptor of poly(ADPR).

Caplan et al. (1979) using a partially purified enzyme preparation containing an endogenous acceptor protein, also showed the ability of the enzyme for self-ADP-ribosylation. By analysing the effect of DNA on the enzyme and its endogenous acceptor, which could be separated from each other on SDS gel electrophoresis, they showed that DNA specifically stimulated ADP-ribosylation of the endogenous acceptor but had no effect on the ADP-ribosylation of the synthetase itself or on the transfer of ADPR to an exogenous acceptor such as H1. Whether these results are compatible with those using 'active' DNA is unknown. The relationship in functional and structural terms of the endogenous acceptor to the synthetase is also not clear at present.

The purification of the synthetase has accelerated the understanding of the function of the enzyme. It has, however, also brought into question the extent to which purification may proceed before the properties of the purified enzyme deviate significantly from those existing in vivo. The contrasting results outlined above and the

discrepancy in molecular weight of the enzyme all suggest the loss of factors during the purification of the enzyme which may be an intrinsic part of the polymer synthesizing system. The synthetic system of poly(ADPR) thus appears to be very complex.

The synthesis of poly(ADPR) is specifically inhibited by various groups of compounds. This inhibition has been used as a probe in an attempt to understand both the function of the synthetase and the cellular processes which it affects. Substrate analogues such as α -dNAD⁺ and NADH have been found to be potent inhibitors, but the relatively high specificity of the enzyme prevents effective inhibition by such analogues as 3-acetylpyridine NAD⁺ (Hilz & Stone, 1976). End-product inhibition by nicotinamide and 5-methylnicotinamide has been demonstrated by Clark et al. (1971) and Preiss et al. (1971). The inhibition was competitive with K_i values of 20 μ M and 200 μ M for nicotinamide and 5-methylnicotinamide, respectively. Competitive inhibition by thymidine and some of its analogues was demonstrated by Preiss et al. (1971); the inhibitory capabilities being similar to those of nicotinamide and its analogues. Although the significance of this finding is not understood Shall (1975) provided evidence which suggested that the inhibition was not a chemical coincidence. More recent results from Levi et al. (1978) demonstrated that methylated xanthines and cytokinins (N⁶ derivatives of adenine) were also good inhibitors, particularly theophylline. However, all the above compounds, as well as inhibiting poly(ADPR) synthetase, are known to affect other

cellular processes. Nicotinamide affects the synthesis of NAD^+ and therefore poly(ADPR). In fact, Bredehorst et al. (1980) showed an increase in mono(ADPR) conjugate levels on nicotinamide administration to rat liver and Berger & Sikorski (1980) demonstrated that nicotinamide stimulated repair of DNA damage, possibly through poly(ADPR), in human lymphocytes. These results demonstrate that nicotinamide, as a precursor of NAD^+ , may enhance ADP-ribosylation. Consequently, the effect of 5-methylnicotinamide, which does not elevate NAD^+ levels, is a better reflection of the inhibition of the synthetase by nicotinamide and its analogues. Thymidine inhibits DNA synthesis by depleting dCTP levels (Meuth et al., 1976). In addition, methylated xanthines and cytokinins affect cyclic AMP phosphodiesterase.

Because of these problems Purnell & Whish (1980a) searched for physiologically significant inhibitors of the synthetase that affect only poly(ADPR) synthesis and that are also able to enter the cell. As a result they showed that various benzamides were very good inhibitors of the synthetase. Shall (1975) was the first to show that benzamide (an analogue of nicotinamide) was a good inhibitor of the synthetase. However, because of its extremely low solubility and very hydrophobic nature it was difficult to use. By using benzamides substituted in the 3-position Purnell & Whish (1980a) showed this group of compounds to be more potent inhibitors than either nicotinamide or thymidine. Inhibition by 3-aminobenzamide and 3-methoxybenzamide was competitive, with K_i values of $1.8\mu\text{M}$ and $1.5\mu\text{M}$ respectively, in pig thymus nuclei. 3-aminobenzoic acid

was also found to be a good inhibitor. The same group also studied the inhibitory effects of acetophenones (Purnell & Whish, 1980b). As in the case of the benzamides the 3-methoxy derivative was most inhibitory. It seems that the degree of inhibition cannot be easily explained in terms of simple structural or electron properties of the molecules. In conclusion, the substituted benzamides 3-aminobenzamide and 3-methoxybenzamide gave greatest inhibition at equimolar concentration to substrate and, in addition, are physiologically specific. Consequently, they will prove useful not only in vitro but, more importantly, may be valuable for in vivo studies.

Recently, Yamamoto & Okamoto (1980) have shown picolinamide (an isomer of nicotinamide) to be an inhibitor of poly(ADPR) synthetase. Inhibition by picolinamide was almost identical to that of nicotinamide but in contrast to nicotinamide the molecule is not a precursor of NAD^+ . Picolinamide protected both the streptozotocin-induced reduction of NAD^+ content and depression of proinsulin synthesis, presumably by blocking the synthesis of poly(ADPR). As a result a relationship between poly(ADPR), NAD^+ content and proinsulin in pancreatic islets was proposed.

Recently, the development of nucleotide-permeable cell systems (Halldorsson et al., 1978; Berger et al., 1978a) has allowed the measurement of poly(ADPR) synthetase activity under conditions which more closely reflect those in vivo than in isolated nuclei. These studies are discussed in Section 1.8.

1.4 DEGRADATION OF POLY(ADPR)

Poly(ADPR) may be cleaved at the pyrophosphate bond or at the ribosyl (1"-2') ribose linkage, the characteristic bond formed between successive ADPR units in the polymer. In addition, the bond between the anomeric carbon on ribose and protein may be cleaved.

The pyrophosphate bond is cleaved by a phosphodiesterase from the venom of the snake Crotalus adamanteus. Analysis of partial degradation products by Sephadex G-50 chromatography revealed that cleavage was endonucleolytic (Matsubara et al., 1970a). The final products of digestion are phosphoribosyl-AMP from the internal residues of the polymer and 5'AMP from the terminus. Snake venom phosphodiesterase has proved to be a very useful tool in the identification and characterization of poly(ADPR) because of the unique product, phosphoribosyl-AMP. Rat liver phosphodiesterase was also reported to cleave the pyrophosphate bond of poly(ADPR) and as such was the first enzyme found to degrade the polymer from animal tissues. Hydrolysis is exonucleolytic (Futai et al., 1968) from the AMP-terminus (Matsubara et al., 1970b), producing first AMP then increasing amounts of phosphoribosyl-AMP with time. The enzyme is also capable of hydrolysing NAD⁺ and ADPR.

The polymer is cleaved at the ribose-ribose bond by the enzyme poly(ADPR) glycohydrolase. The enzyme was first discovered by Miwa & Sugimura (1971) in calf thymus nuclei. The hydrolysis product was identified as ADPR by its elution position on Dowex chromatography and its inability to be converted to ribosyl-adenosine on alkaline phosphatase

digestion. Analysis of various nuclear fractions revealed that the enzyme may exist in both soluble and chromatin-bound forms, half the activity being dissociated from chromatin at 0.15M NaCl. As such the enzyme seems to be relatively loosely bound to chromatin compared to the tight association of the synthetase which requires 1M NaCl for half dissociation (Miyakawa et al., 1972). The glycohydrolase appears not to cleave the linkage between ADPR and protein since little hydrolysis of histone-bound mono- (ADPR) was observed (Ueda et al., 1972). This observation suggests that an additional enzyme, a 'hydrolase', is needed to cleave this last residue from protein (see below). In addition, the enzyme does not cleave the ribose-ribose linkage of phosphoribosyl-AMP, possibly suggesting that a certain conformation or linkage may be required for recognition. Poly(ADPR) glycohydrolase is sensitive to ionic strength, being completely inhibited by 0.5M $(\text{NH}_4)_2\text{SO}_4$ (Stone et al., 1973; Miyakawa et al., 1972). It is also inhibited, non-competitively, by cyclic AMP with an apparent K_i of 1.5mM in rat liver (Ueda et al., 1972; Miyakawa et al., 1972; Stone et al., 1973) and by its end-product, ADPR (Miyakawa et al., 1972). Inhibition by core histones is also apparent but this may be reversed by addition of DNA (Miwa et al., 1974).

Purification of the glycohydrolase has confirmed these properties and enabled further characterization of the reaction. A 200-fold purification of the enzyme with 4% yield from calf thymus has been carried out (Miwa et al., 1974) and also a partial purification from Physarum polycephalum (Tanaka et al., 1976). The optimum pH of the

enzyme varies between 6.0 and 7.5 for Physarum polycephalum and calf thymus, respectively. The molecular weight is approximately 50,000 and the enzyme seems to require SH groups for activity. The K_m value for the polymer in calf thymus is 0.58 μ M. Burzio et al. (1976) showed the existence of two forms of glycohydrolase in rat testis which separated on phosphocellulose chromatography and appeared to reside mainly in the soluble fraction of the cell in contrast to other tissues. The properties of the enzyme were very similar both to each other and to other glycohydrolases. The relationship between the two variants is uncertain and although it was shown to be unlikely that one was a proteolytic product of the other, it may be that a non-protein molecule which is necessary for some aspect of its function has been lost.

Miwa et al. (1974) demonstrated that the mode of action of the glycohydrolase was exoglycosidic. By performing a two-step hydrolysis of poly(ADPR), using purified glycohydrolase followed by snake venom phosphodiesterase, they showed that the number of ADPR moieties released per chain was equal to the number of ribose-ribose bonds split.

The reported K_m values of 0.58 μ M and 28 μ M for poly(ADPR) glycohydrolase and phosphodiesterase, respectively, and the fact that glycohydrolase action, but not phosphodiesterase, leaves the polymer in a form capable of re-elongation, suggested that the glycohydrolase is the metabolically significant enzyme for poly(ADPR) degradation. Miwa et al. (1975) provided additional evidence in support

of this suggestion by analysing the degradation of poly(ADPR) in the presence of inhibitors of glycohydrolase and phosphodiesterase. Hydrolysis was inhibited in the presence of cAMP but was similar in the presence or absence of NaF, in a number of different normal and tumour tissues. In addition, the product of glycohydrolase, ADPR, was rapidly hydrolysed in liver homogenate, while the product of phosphodiesterase, PR-AMP, was not metabolized any further.

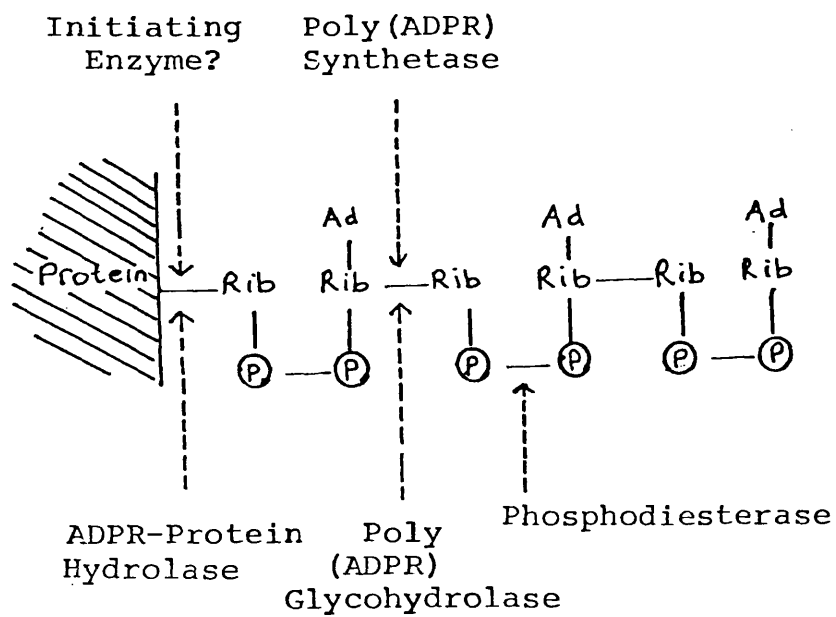
In contrast to its effect on poly(ADPR) synthetase DNA has been shown to inhibit glycohydrolase activity (Hayaishi & Ueda, 1974; Miwa et al., 1974; Burzio et al., 1976; Stone et al., 1978). This effect was specific to denatured, i.e. single stranded, rather than native DNA. Stone et al. (1978) showed that the enzyme bound more tightly to denatured DNA-cellulose than to double-stranded DNA-cellulose, thus supporting this finding. In addition, the inhibition could be overcome either by adding an equal mass of H1 or by increasing the ionic strength of the medium. The activity of poly(ADPR) glycohydrolase may be affected by changes in the extent to which proteins are bound to DNA; when DNA is in excess over proteins, the glycohydrolase binds to the DNA and is inhibited.

Miwa et al. (1975) carried out a comprehensive analysis of poly(ADPR) glycohydrolase activity in a number of different normal and malignant tissues. They showed that of all tissues testis had the highest degradative activity. This result was supported by the results of Burzio et al. (1976). Activity was also quite high in

kidney, liver and intestinal mucosa. The activity in rapidly growing hepatomas was higher than in normal liver whereas in slowly growing hepatomas it was much the same. This difference was not apparent, however, if the activity was expressed as per mg of DNA rather than per mg of protein. Whether these results are significant in connection with the metabolism of poly(ADPR) in vivo remains to be elucidated. In fact, Lorimer et al. (1977) showed that in various nuclear preparations the chain length of ADP-ribosyl histone H1 produced was inversely related to the level of poly(ADPR) degrading activity. Thus, it will prove interesting if a relationship between poly(ADPR) synthetase and glycohydrolase activities is seen during the different nuclear events of the cell. In addition, it is important when attempting to determine the extent of ADP-ribosylation in vitro or in vivo, to realize that the conditions used will govern the relative synthetic and degradative activities present; conditions for in vitro analysis are usually optimal for synthesizing activity and isolation of in vivo material has often been carried out under conditions which may activate glyhydro-lase e.g. 0.15M NaCl.

Since poly(ADPR) glycohydrolase does not appear to cleave the last ADPR residue from protein it was realized that a 'hydrolase' may exist. Okayama et al. (1978) showed the existence of such an enzyme in rat liver. The hydrolase was isolated from glycohydrolase by ammonium sulphate precipitation and DEAE-cellulose chromatography. Analysis of the reaction product by paper and Dowex-1 chromatography indicated that although the split product

Fig. 1.4 Schematic Representation of Poly(ADPR)-Protein Metabolism



contained ADPR it was not identical to it. Evidence suggested that it was either an altered ADPR molecule, or ADPR linked to some unidentified compound. This finding suggests that the ADPR might not be directly attached to the amino acid at the acceptor site of the protein. The enzyme had a pH optimum of 6 and a molecular weight of approximately 240,000. ADPR and DNA were strong inhibitors (Ueda et al., 1980). Recent results have shown that it may split both the bond between mono(ADPR) and various proteins and those of oligo or poly(ADP-ribosyl) proteins. The significance of this result with respect to the dynamics of poly(ADP-ribosyl) protein metabolism in chromatin remains to be elucidated.

The synthesis and degradation of poly(ADPR)-protein is represented schematically in Fig. 1.4.

1.5 QUANTITATION

Determination of the level of poly(ADPR) in the cell is most relevant to the study of its function; variation in poly(ADPR) content under a variety of conditions may reflect its role in different processes. Adenine-labelled NAD^+ can be successfully used in incubations with isolated nuclei to determine the acid-insoluble radioactivity in ADPR in vitro and, as such, is a convenient method for the quantitation of polymer under these conditions. However, determination of poly(ADPR) content in intact cells as well as proof of its natural occurrence are difficult to obtain because of the lack of specific precursors able to permeate the cell membrane. $[^3\text{H}]\text{NAD}^+$, although the

immediate substrate of poly(ADPR) synthetase, cannot penetrate the cell membrane intact but is degraded by extracellular and pericellular enzymes to nicotinamide and adenosine (Nolde & Hilz, 1972). Furthermore, the low concentrations of poly(ADPR) in the cell, the difficulty in achieving quantitative separation from nucleic acids and the high turnover of the molecule necessitate special procedures and controls in order to obtain meaningful results. In vitro experiments indicate considerable heterogeneity in poly(ADPR) both in terms of chain length and linkage to protein. The quantitation and analysis of poly(ADPR) in vivo is therefore very pertinent to the elucidation of the role of this protein-modifying system in nuclear and cellular function.

Stone & Hilz (1975) were the first to attempt an accurate measurement of endogenous levels of ADPR, using an isotope dilution procedure. They chose to analyse levels of mono(ADPR). To avoid destruction of ADPR residues during release from proteins, material was incubated with neutral hydroxylamine rather than NaOH (Goebel et al., 1977). By a combination of ion-exchange and paper chromatography, followed by enzyme digestion, they were able to quantitate mono(ADPR) residues in a number of different tissues. Addition of [³H]ADPR allowed these values to be independent of losses incurred during the many purification steps necessary to achieve constant specific radioactivity. From the final specific activity and knowledge of the starting specific activity, in vivo mono(ADPR) levels of 5.28 and 2.19nmol of ADPR/mg

of DNA were obtained for adult and neonatal rat liver, respectively; levels in rapidly proliferating tissues seemed to be about 2.5x lower than those in adult tissues. Although quite accurate this method is limited to the quantitation of hydroxylamine-sensitive mono(ADPR) residues, no value for NH_2OH -resistant residues being obtained (Adamietz & Hilz, 1976). A similar method was used for the determination of poly(ADPR) levels (Stone et al., 1976), the polymer being converted to phosphoribosyl-AMP and then ribosyl-adenosine. Values of 5.59nmol and 6.32nmol/mg of DNA were obtained for adult and neonatal rat liver, respectively. Levels of poly(ADPR), in contrast to mono(ADPR), did not seem to vary with proliferation rate of the tissues. A comparison of levels in adult tissue, though, showed that mono and poly(ADPR) seemed to be present in similar quantities.

Goebel et al. (1977) developed an optical test for the quantitation of both total, i.e., hydroxylamine-sensitive and -resistant, mono(ADPR) and poly(ADPR). Additional advantages of the test were speed and the need for much less material than the isotope dilution procedures. The method relies on the unique property of ADPR to yield 5'AMP when subjected to alkaline treatment. The 5'AMP released was quantitated by using a linked enzyme assay. Poly(ADPR) could be quantitated by prior digestion with poly(ADPR) glycohydrolase. The average chain length of the polymer could also be determined by measuring the ratio of nmol of ADPR released by glycohydrolase to nmol of 5'AMP released by phosphodiesterase; in vitro analysis showed similar

values to those obtained using the conventional method of Nishizuka et al. (1969). The method of Goebel et al. (1977) has the advantage, however, that it may be applied to unlabelled material. Nanomole levels of ADPR could be detected.

Niedergang et al. (1978) used an alternative to relying on radioactive labelling of poly(ADPR) by developing a fluorimetric assay. Rat liver nuclei were treated with alkali and the released poly(ADPR) purified by a four step procedure. After enzymic hydrolysis by snake venom phosphodiesterase, PR-AMP was quantitated fluorimetrically by a modification of the glyoxal method of Yuki et al. (1972). ADPR residues in the range 30 to 300pmol could be determined. A value of 3.35nmol ADPR/mg DNA was obtained. This method did not detect mono(ADPR) residues, however, because under the alkaline conditions employed, 75% of these residues are converted to 5'AMP (Goebel et al., 1977). Sims et al. (1979) also employed a fluorimetric method for the determination of in vivo poly(ADPR) levels. The method was based on the dihydroxy-borate column of Okayama et al. (1978) and as such allowed a more specific isolation of poly(ADPR) from other nuclear components than the method of Niedergang et al. (1978). Poly(ADPR), isolated from SV40 virus-transformed 3T3 cells (SVT2), was enzymically converted to ribosyl-adenosine which was then converted to a 1, N⁶-etheno derivative by treatment with chloroacetaldehyde. After purification by high-pressure liquid chromatography the derivative was quantitated fluorimetrically. However, only 65% recovery

of ribosyladenosine was obtained by this method (Juarez-Salinas et al., 1979). Although both these fluorimetric determinations allow quantitation at picomole level, and as such are more accurate than the methods of Hilz's group, they are only able to detect poly(ADPR) levels; levels of mono(ADPR) may contribute significantly to ADP-ribosylation in vivo (Stone et al., 1976).

The possible quantitation of in vivo poly(ADPR) levels has been greatly enhanced by the discovery of antibodies raised against poly(ADPR). Kanai et al. (1974) were the first to report on such a phenomenon when they injected rabbits with a complex of poly(ADPR) and methylated bovine serum albumin. The preparation was very specific and could detect poly(ADPR) at 3µg/ml. The natural occurrence of poly(ADPR) antibodies was demonstrated by the same group in patients with the autoimmune disease systemic lupus erythematosus (Kanai et al., 1977). Okolie & Shall (1979) suggested that the production of such antibodies may prove useful as a diagnostic test for the disease. Kanai et al. (1978) showed that poly(A)·poly(U) or structurally-related double-stranded RNA may have some role in the production of antibodies to poly(ADPR) in these patients. They later went on to show that antibody against oligo(ADP-ribosyl)ated histone might exist in SLE patients in addition to free poly(ADPR) (Kanai et al., 1980). The production of antibodies to poly(ADPR) allowed the development of radioimmunoassay as a method of quantitation of polymer levels in vivo. Sakura et al. (1977) estimated calf thymus to contain about 0.02µg/mg DNA of poly(ADPR).

Kidwell & Mage (1976) used radioimmunoassay to determine amounts of poly(ADPR) during the cell cycle of synchronized HeLa cells. They found, in agreement with pulse-labelling techniques, that the amount of polymer peaked at mid S-phase with a second even larger increase at the S-G2 transition point. Ferro et al. (1978) used antibodies against poly(ADPR)_{4.0} to determine larger than tetrameric poly(ADPR). Nuclei were isolated by an organic solvent technique, thus minimizing the effect of degradative enzymes. Poly(ADPR) levels in rat liver and pigeon heart were 58ng/mg DNA and 1µg/mg DNA, respectively. In addition, it was found that PR-AMP could inhibit binding of [¹⁴C]-poly(ADPR) ten times more effectively than ADPR, suggesting that the ribose-ribose bond in the poly(ADPR) immunogen is important in determining the specificity of the antibody. Values obtained were significantly lower than those determined by fluorimetric or isotope dilution methods, possibly suggesting that the majority of poly(ADPR) in vivo exists as small chains. Although quantitative, these radioimmunoassays are limited in that they are only able to measure poly(ADPR) of a minimum chain length of 4. In addition, the chain length itself was shown to affect the radioimmunoassay; the shorter the chain length of polymer, the greater the amount of ADPR required for 50% inhibition (Sakura et al., 1977). Since short chain poly(ADPR) may contribute significantly to ADP-ribosylation in vivo, Sakura et al. (1978) developed an antibody against 2'-(5"-phosphoribosyl)-5'AMP and so allowed the quantitation of these species. Inhibition experiments showed that

the adenine ring, 5'-phosphate residue and ribose-ribose bond of PR-AMP were essential for the antigenic determinant; this finding thus confirmed the results of Ferro et al. (1978). The anti-PR-AMP antibody bound not only PR-AMP but also poly(ADPR) of various chain lengths. However, ADPR, an isomer of PR-AMP, was 3,000x less effective than PR-AMP; the assay was still limited in that it could not quantitate mono(ADPR) levels.

Bredehorst et al. (1978a,b) developed a radioimmunoassay which allowed not only the quantitation of protein-bound residues, but both mono and poly(ADPR) levels could be determined. The assay relied on the high specificity of alkaline conversion of ADPR to 5'AMP and the high selectivity of the 5'AMP-specific antibodies produced. Poly(ADPR) could be determined by prior degradation to ADPR by poly(ADPR) glycohydrolase. This method allowed quantitation in the range 1-40pmol in the presence of large quantities of nucleic acids or 3'AMP. Using the 5'AMP-specific radioimmunoassay Bredehorst et al. (1978c) were able to determine the occurrence of mono(ADPR)-protein conjugates linked by hydroxylamine-sensitive and resistant bonds in Ehrlich ascites tumour (EAT) cells (stationary phase) and rat liver. NH_2OH -resistant ADPR-protein conjugates could be determined by the difference in levels produced after cleavage with NH_2OH and NaOH . Total, i.e. NaOH released, mono(ADPR) residues in EAT cells and rat liver were 1.66nmol/mg DNA and 12.6nmol/mg DNA, respectively. NH_2OH -sensitive values were 0.48nmol/mg DNA and 5.3nmol/mg DNA for EAT and rat liver, respectively.

These results showed that, contrary to the results in vitro, more protein-ADPR linkages were resistant to NH_2OH than sensitive. Whether this difference could be partly explained by the fact that only mono(ADPR) levels have been determined by the radioimmunoassay will be evident on analysis of hydroxylamine-sensitive and -resistant poly(ADPR). This analysis, however, would be complicated by chain length differences. It must also be mentioned that alkaline conversion of ADPR to 5'AMP is not quantitative, only 75% of ADPR being specifically converted to 5'AMP (Goebel et al., 1978b). It seems that only with the development of a radioimmunoassay specific for ADPR will quantitation of total protein-bound ADPR in the cell be possible. So far, however, attempts have proved difficult; significant titres of ADPR antibodies could only be obtained with cm^6 ADPR coupled to methylated serum albumin. However, using this method, a similarly high production of 5'AMP antibodies was also obtained, presumably due to hydrolytic breakdown of the ADPR antigen in the recipient animals (Bredehorst et al., 1978a). The results above have shown that in vivo ADP-ribosylation may vary considerably from that in vitro and thus stresses the importance of in vivo studies.

1.6 IN VIVO STUDIES

Doly and Mandel (1967) were the first to report on the natural occurrence of poly(ADPR). Chickens were injected with $[^3\text{P}]$ and putative purified poly(ADPR) from

liver analysed by digestion with snake venom phosphodiesterase. The [^{32}P]-labelled in vivo products co-chromatographed with authentic in vitro products (PR-AMP and AMP), thus indicating the natural occurrence of the polymer.

However, the system by which nuclear proteins are ADP-ribosylated appears to be complex. This, together with the fact that most studies to date have been carried out in vitro where the true properties of the ADPR-protein complex may be altered contributes significantly to the fact that no clear-cut function has yet been assigned to this protein modification. The analysis of ADP-ribosylation in vivo, however, is complicated by the fact that NAD^+ cannot enter cells and, as such, no specific precursor exists for the detection of ADPR. In addition, levels of poly(ADPR) in the cell are low compared with those of other nuclear macromolecules. The determination of the existence of poly(ADPR) in vivo is also seriously hampered by the fact that little is known about its native status in the cell i.e. its interaction with the chromatin. Experiments performed in vitro suggest considerable heterogeneity with respect to both the structure and function of poly(ADPR). An accurate and simple analytical system for protein ADP-ribosylation in vivo is clearly necessary.

Quantitation methods developed for mono(ADPR) and poly(ADPR) have demonstrated the natural occurrence of these molecules. They have also allowed a preliminary comparison of ADP-ribosylation in vitro and in vivo to

be made. Sakura et al. (1977) investigated the chain length distribution of poly(ADPR) in vivo and showed that it may be as heterogeneous as that in vitro. Ferro et al. (1978) showed that mono(ADP-ribosyl)ation may contribute to the majority of ADP-ribosylation in vivo, contrary to in vitro studies where much higher chain lengths are normally found. Bredehorst et al. (1978c) compared levels of NH₂OH-sensitive and NH₂OH-resistant ADPR-protein conjugates in EAT cells and rat liver by a highly sensitive radioimmunoassay. They showed that a greater percentage of ADPR-protein conjugates in vivo may be resistant to NH₂OH than in vitro.

Farzaneh & Pearson (1978), while studying the synthesis of poly(ADPR) by nuclei isolated from Xenopus laevis embryos at different stages of development, showed that chain length determination by hydroxyapatite chromatography generally gave higher values than when material was analysed by the conventional method of Nishizuka et al. (1969). These results suggested that poly(ADPR) synthesized in vitro is a covalent elongation of pre-existing chains in vivo and, as such, demonstrated the existence of ADP-ribosylation in vivo.

More recently Caplan et al. (1980), using an assay for chemical quantities of poly(ADPR), have analysed levels of the polymer in vivo as a function of chick limb mesenchymal cell development. They showed that a correlation between cellular poly(ADPR) levels and the early phases of mesenchymal cell differentiation and development may exist. In addition, Olivera et al. (1980)

have developed a red blood cell fusion technique where red blood cells loaded with NAD^+ (which is not degraded) are fused to HeLa cells, which allows the investigation of mono(ADP-ribosyl)ation or poly(ADP-ribosyl)ation events in vivo.

Additional, indirect evidence in support of the natural occurrence of poly(ADPR) comes from two observations: (i) the existence of enzymes for the specific synthesis and degradation of the polymer, and (ii) the natural occurrence of anti-poly(ADPR) antibody in human sera of patients with systemic lupus erythematosus.

However, since the action of poly(ADPR) is mediated through the covalent modification of nuclear proteins, it seems that conclusive evidence for the natural occurrence of this modification will only arise from the isolation of protein ADP-ribosylated in vivo. Analysis of the number and types of protein modified may thus give a good indication of the role of ADP-ribosylation in nuclear function.

Dietrich et al. (1973) analysed ADPR residues rendered acid-soluble by NH_2OH treatment in rat liver nuclei radiolabelled with $[^3\text{P}]$. They tentatively identified ADPR by chromatographic separation of the released material and thus proposed the existence of in vivo bound ADPR. Employing this procedure they were unable to detect the presence of poly(ADPR). However, degradation of poly(ADPR) may have occurred during isolation of nuclei. Smith & Stocken (1973) used the same precursor and a similar isolation procedure to demonstrate that histone H1 was ADP-ribosylated in vivo

via serine phosphate. The ADP-ribosylation of non-histone protein P1 and a small basic polypeptide of molecular weight 3,000, which were co-extracted with histone H1, was also demonstrated (Smith & Stocken, 1975).

Ueda et al. (1975) took advantage of the fact that studies in vitro suggested that ADPR is principally bound to histones. They injected [^{14}C]ribose and [^3H]adenine intraperitoneally into rats and extracted liver histones with 0.25M HCl. Carboxymethyl-cellulose chromatography of this material revealed that the radioactivity co-chromatographed with histone H1 and to a lesser extent with the other histones. Part of the protein-bound radioactivity was rendered acid-soluble by treatment with snake venom phosphodiesterase or neutral NH_2OH . 5'AMP and PR-AMP were recovered from the enzyme digest and ADPR from the NH_2OH treatment. Average chain lengths of 2 to 5 were obtained. These results were the first to show conclusively the existence of ADP-ribosylation in vivo by the identification of the unique product PR-AMP. The incomplete degradation of poly(ADPR) by snake venom phosphodiesterase and the lesser degree of susceptibility to NH_2OH compared to that in vitro may reflect a fundamental difference between in vivo and in vitro material.

Young & Sweeney (1978) were able to isolate ADP-ribosylated proteins from unfertilized mouse ova labelled with [^3H]adenosine. Since the ova were not actively synthesizing DNA or RNA during the labelling period, all the acid-insoluble radio-labelled material was present

as poly(A) or ADP-ribosylated protein. The maximum incorporation of [^3H]adenosine was between two and five hours after ovulation. The retention of [^3H]adenosine-labelled material in the organic phase after chloroform-phenol extraction of unfertilized ova lysates and the presence of alkali-resistant labelled material in these lysates suggested that [^3H]adenosine was incorporated into a non-RNA component which was associated with protein. Snake venom phosphodiesterase digestion of this material, which had been previously treated with Pronase, revealed the presence of both 5'AMP and PR-AMP on analysis in a number of thin-layer chromatographic systems. Snake venom phosphodiesterase digestion without prior degradation with Pronase did not release all the material into the supernatant of an ethanol/sodium acetate wash. This suggested the possibility of steric hinderance by protein. Most of the acid-soluble material released was in the form of 5'AMP, indicating a preponderance of mono(ADP-ribosyl)ated protein. Incubation of putative ADP-ribosylated protein with hydroxylamine or alkali released some of the acid-insoluble material, which was identified as oligo(ADPR). Mono(ADPR), however, did not seem to be released from protein on treatment with acid (Smith & Stocken, 1973), alkali or hydroxylamine (Nishizuka et al., 1969); no ADPR could be detected on treatment with these reagents. Since the ovulated ovum does not divide, synthesize DNA or RNA, or undergo chromatin condensation, the ADP-ribosylation of unfertilized ovum protein(s) may be associated with the modification of enzyme activity.

The existence of ADP-ribosylated protein was also shown in the mouse one-cell embryo (Young & Sweeney, 1979). Incorporation of label was high immediately after fertilization. The chain length of the poly(ADPR) synthesized appeared to be 2. Soon after fertilization chromatin of both the ovum and the fertilizing sperm changes from a highly condensed to a diffused state, DNA is replicated and a change occurs in the pattern of proteins synthesized. Modification of proteins in the newly fertilized ovum by ADP-ribosylation may therefore play a role in these changes.

Adamietz et al. (1978) attempted to demonstrate fundamental differences in ADP-ribosylation processes in vivo and in vitro by comparing (ADPR)_n-histone H1 conjugates of HeLa cells grown for 24 hours in [³H] adenosine with those synthesized from [³H]NAD⁺ by isolated HeLa nuclei. This is the most direct comparison made in an attempt to solve the differences in ADP-ribosylation. To ensure that such a comparison could be made it was necessary to isolate histone H1 and its ADP-ribosylated derivatives free of any contaminants, including high-mobility group (HMG) proteins. Such a vigorous purification had not previously been employed (Smith & Stocken, 1973; Ueda et al., 1975). Purification was achieved by repeated 5% (w/v) perchloric acid extraction followed by cation-exchange chromatography on Biorex-70 in the presence of guanidium hydrochloride and urea. This allowed removal of contaminants in the crude H1 preparation; H1 itself was quantitatively eluted from the column by increasing

the concentration of guanidium chloride. Both qualitative and quantitative differences were seen in ADP-ribosylated histone H1 formed under the two conditions. Chain length analyses by chromatography of the products of snake venom phosphodiesterase and poly(ADPR) glycohydrolase digests, previously freed from protein by alkali, revealed that most (ADPR)_n-histone H1 conjugates formed in vivo carried single ADPR residues, less than one quarter of the total ADPR residues being in the form of oligomeric or polymeric chains. This means that approximately 90% of acceptor sites are occupied by mono(ADPR). In contrast, isolated HeLa nuclei formed ADP-ribosylated histone H1 which contained predominantly polymeric ADPR residues. Analysis of protein-ADPR conjugates by gel electrophoresis and caesium sulphate density gradient centrifugation confirmed these differences. Also, although 'H1 dimer' (Stone et al., 1977) was apparent on gel electrophoresis of in vitro material, no such dimer could be detected in vivo.

Additional qualitative differences could be seen with respect to the nature of the ADPR-protein linkage. Alkali treatment (pH 10.5) liberated most, but not all, of the ADPR residues of in vivo ADP-ribosylated histone H1; 20-30% remained bound to protein as revealed by CM-cellulose chromatography. This may indicate the existence of a new type of linkage in conjugates isolated from intact tissue. In addition, no significant amount of (ADPR)_n were released by neutral NH₂OH treatment and no lability of (ADPR)_n-histone H1 conjugates towards moderate acidic conditions was observed. These results agree with those of Young & Sweeney (1978).

In contrast, in vitro formed histone H1-ADPR residues were linked by NH₂OH-sensitive and NH₂OH-resistant, alkali-labile bonds.

Quantitative analysis revealed that an estimated 1620pmol ADPR/mg histone H1 and 218pmol ADPR/mg histone H1 existed in vitro and in vivo, respectively. The content of histone H1-bound ADPR in vivo therefore seemed to be about one eighth of that in vitro.

The macromolecular association of immunoreactive, naturally occurring poly(ADPR)_n, with histones and non-histone proteins has been determined by Minaga et al. (1978). This was achieved with the aid of an improved method of extraction of poly(ADPR) and a combination of radioimmunoassay and molecular filtration. Greater than 99% of naturally occurring poly(ADPR)_n appeared to be associated with non-histone proteins, less than 1% being distributed amongst the histone fractions. The chain length of the polymer varied between 4 and 34. The covalent association of poly(ADPR) with non-histone proteins was demonstrated by dihydroxyboronate affinity chromatography.

The data obtained on analysis of in vivo poly(ADPR) and ADP-ribosylation of proteins reveal fundamental differences in terms of chain length, linkage to protein and type of protein modified to that in vitro. These results clearly indicate that conclusions drawn from experiments with isolated nuclei or chromatin may not be valid for intact tissue. Apparently, new methods must be devised for the isolation and analysis of acceptor proteins

ADP-ribosylated in vivo if the true functions of this protein modification are to be revealed.

Analysis of the effect of known inhibitors of poly-(ADPR) synthetase supports this proposal. Ferro et al. (1978) showed that nicotinamide and nicotinic acid when administered in vivo augmented the (ADPR)_n content of rat liver and heart. It may be that local concentration effects on chromatin differ in vivo from those in vitro, resulting in conditions which favour NAD⁺ synthesis rather than the inhibition of poly(ADPR) synthetase. Tanuma et al. (1979), on studying the effect of thymidine as a cell synchronizing agent and its effect on poly(ADPR) synthesis, revealed that nuclei isolated from cells immediately after thymidine synchronization exhibited a significantly increased amount of poly(ADP-ribosyl)ation. A similar effect was seen with nicotinamide but not with hydroxyurea and amethopterin. This increase was shown not to be a result of increased poly(ADPR) synthetase activity but rather due to the increase of acceptor sites for poly(ADP-ribosyl)ation. Bredehorst et al. (1980) extended these studies by analysing the increase in mono(ADPR)-protein conjugate levels in rat liver induced by nicotinamide administration. They found that an increase in total mono(ADPR) residues occurred on nicotinamide administration, thus eliminating the possibility that increased ADP-ribosylation was a result of chain elongation only. These results support the proposal of Tanuma et al. (1979) who suggested that the rise is due to an increase in acceptor sites or proteins for ADP-ribosylation. On

analysis of the NH_2OH -sensitive and NH_2OH -resistant fractions, however, Bredehorst et al. (1980) showed that the NH_2OH -sensitive conjugates were significantly more affected than the resistant subfraction. This result is not easily explained by a simple causal relationship; it may be that only one of the subfractions eventually fits such a correlation. An interesting effect of nicotinamide on the repair of DNA damage in human lymphocytes was shown by Berger & Sikorski (1980a). Nicotinamide was found to stimulate the amount of repair synthesis in cells subjected to DNA damage, reaching a maximum at 2-5mM nicotinamide. In contrast, DNA synthesis in cells not subjected to DNA damage was inhibited by these concentrations. Poly(ADPR) may play a role in DNA repair (see Section 1.8). Whether this effect is mediated through poly(ADPR) and the availability of acceptors for ADPR remains to be elucidated. It does seem, though, that cells not stimulated in repair synthesis by nicotinamide may be depleted of a factor required for continuation of DNA repair. The complexity of these results in comparison with those in vitro again supports the need for the development of new methods for analysis of ADP-ribosylation in vivo.

1.7 ADP-RIBOSYLATION OF NUCLEAR PROTEINS

The existence of poly(ADPR) bound to proteins was first observed by Nishizuka et al. (1968) and the covalent nature of this linkage was demonstrated using caesium chloride density gradient centrifugation in the presence

of guanidium chloride, by Otake et al. (1969).

The nature of this linkage has been analysed by its susceptibility to various reagents. Nishizuka et al. (1969) analysed the pH stability of the bond and showed, on the basis of release of acid-soluble radioactivity, that under alkaline conditions the linkage is extremely labile, the half-life in 0.1M NaOH at 0°C being approximately five minutes. The linkage was also labile in neutral hydroxylamine but appeared to be quite stable under acidic conditions. Adamietz & Hilz (1976) extended these studies to analyse the susceptibility of long chain poly(ADPR) which per se is acid-insoluble. By carrying out caesium sulphate density gradient centrifugation in the presence of highly dissociating concentrations of urea and guanidium chloride (since long chains tended to form aggregates with proteins) they showed that alkaline treatment cleaved all bonds linking long as well as short chains to nuclear proteins. However, NH_2OH did not release the same amount of $(\text{ADPR})_n$ residues as NaOH; 10-30% of chains appeared to be bound to proteins by an NH_2OH -resistant bond. This result was evident on kinetic analysis and caesium sulphate density gradient centrifugation of material from isolated nuclei of both rat liver and EAT cells. This suggested that ADPR is covalently linked to nuclear proteins by two types of bonds, one being susceptible to alkali and hydroxylamine, the other being alkali-labile but hydroxylamine-resistant. The properties of the former linkage are compatible with the supposition that ADPR may be transferred to a carboxyl

group in the protein molecule. In fact, the susceptibility of the bond is of the same order as the ester-bond in amino-acyl tRNA; the linkage of ADPR to protein may be via a glutamic acid residue.

Dietrich & Siebert (1973), on the basis of hydroxyapatite chromatography of NH_2OH -treated material, suggested that most of the material sensitive to NH_2OH was monomeric. In addition, they showed that two different K_m values were apparent for the formation of NH_2OH -sensitive and NH_2OH -resistant residues which might be indicative of two separate enzyme systems.

Smith & Stocken (1973,1975) analysed the linkage in ADP-ribosylated histone H1. The molecule was degraded by proteolytic enzymes and the nucleotide-containing peptides isolated by ion-exchange chromatography. From the composition of the serine phosphate-associated nucleotide they proposed that ADPR is glycosidically linked to a phosphoserine residue of histone H1. This bond was alkali-and acid-labile but resistant to hydroxylamine. These results contrast with those of Adamietz & Hilz (1976).

In an attempt to clarify this Bredehorst et al. (1978c), using a highly specific radioimmunoassay, compared levels of the hydroxylamine-sensitive and -resistant bonds of mono(ADPR)-protein in intact tissue with those obtained in vitro. Both rat liver and EAT cells exhibited higher levels of ADPR residues bound to acceptors by the NH_2OH -resistant linkage than residues linked by NH_2OH -sensitive bonds. These ratios are the opposite to those obtained

in vitro. A direct comparison of (ADPR)_n-protein conjugates in vivo and in vitro was made by Adamietz et al. (1978) using a highly purified preparation of ADP-ribosylated histone H1. Contrary to results obtained in vitro, (ADPR)_n linked to histone H1 in vivo was not released by neutral hydroxylamine to a significant extent. In addition, alkali treatment (pH 10.5) liberated most, but not all, of the ADPR residues; 20-30% remained bound to protein. Complete cleavage of these residual conjugates was only achieved by treatment with 0.33M NaOH at 56°C for 6 hours, though with destruction of the poly(ADPR) residues. These results may indicate the existence of a new type of linkage so far found only in conjugates isolated from intact tissue. No lability of the (ADPR)_n-histone H1 conjugates towards moderate acidic conditions (0.3M HCl, 30°C, 15 minutes) was observed, suggesting that the serine phosphate linkage observed by Smith & Stocken (1973, 1975) does not exist in histone H1 conjugates in vivo. These more recent results of Hilz's group demonstrate the importance of developing techniques for analysis of proteins ADP-ribosylated in vivo, since significant differences do occur in vitro and in vivo. The lability of the ADPR-protein linkage at neutral or alkaline pH therefore limits the conditions under which ADP-ribosylation of nuclear proteins may be studied; acidic conditions must be employed if this bond is to remain intact.

Using rat liver nuclei two groups have attempted to locate the site or sites at which histones are ADP-ribosylated. On the basis of proteolytic digestion and

the instability of the ADPR-histone H1 linkage to neutral hydroxylamine and alkali, Riquelme et al. (1979a) suggested that the H1 molecule is ADP-ribosylated at the glutamic acid residues in position 2 and 116 of the polypeptide chain and, furthermore, that the glutamic acid at position 2 of the histone H2B molecule is also ADP-ribosylated (Riquelme et al. (1979b)). However, these workers pointed out that the precise location of the site of ADP-ribosylation is hindered by the lability of the bond at pH values necessary for proteolytic degradation and also by the heterogeneity of the modification. Ogata et al. (1980) carried out similar analyses and showed that ADPR was linked to histones H1 and H2B by ester bonds involving either the γ -carboxyl group (Glu², Glu¹⁴, Glu¹¹⁶ of H1 and Glu² of H2B) or the α -carboxyl group (COOH-terminal lysine of H1). These results confirmed and extended those of Riquelme et al. (1979a,b). These workers also proposed that, since all the ADP-ribosylation sites found on histones were located in the polar regions of the molecules that interact with DNA, the modification may profoundly influence chromatin structure. In fact, Burzio et al. (1980) showed that ADP-ribosylation of histone H1 greatly affected its binding to DNA and, in addition, induced conformational changes in the H1 molecule itself. Whether the sites of modification reported by these two groups are a true reflection of the situation in intact cells will only become apparent on isolation of proteins ADP-ribosylated in vivo.

The identification of those proteins modified by ADPR has largely been carried out by the isolation of specific proteins from nuclei which have been labelled with [^3H] or [^{14}C]NAD $^+$. Such an approach has been limited to the analysis of fairly well-defined proteins, particularly the histones. In this way histones H1 and H2B were shown to be the major acceptors amongst the histones (Nishizuka et al., 1968; Hilz et al., 1974; Ord & Stocken, 1977; Poirier & Savard, 1980; Riquelme et al., 1979a,b). In addition, HMG proteins (Caplan & Rosenberg, 1975), trout-specific H6 protein and protamines (Wong et al., 1977) have been shown to be modified. Tanuma et al. (1977) noticed that, on extraction of histones followed by acetic acid-urea polyacrylamide gel electrophoresis the bulk of radioactivity migrated just slower but separated from histone H1. Stone et al. (1977) observed the same phenomenon, characterized the material and showed it to be a 'dimer' complex consisting of two molecules of histone H1 joined by a single chain of poly(ADPR) 15 ADPR units in length. The complex was covalently linked to one H1 molecule and strongly associated with the other.

Several reports have demonstrated ADP-ribosylation of non-histone proteins in addition to histones (Smith & Stocken, 1973; Dietrich & Siebert, 1974; Rickwood et al., 1977; Giri et al., 1978; Lichtenwalner & Sulhaldolnik, 1979). Dietrich & Siebert (1974) showed that the proportion of labelled ADPR on histones or non-histone proteins varied according to the isolation procedure employed. This demonstrates the importance of

assessing the 'purity' of protein fractions once they have been isolated. Rickwood et al. (1977) carried out a broader study of ADP-ribosylation of nuclear proteins. [^{32}P]ADP-ribosylated proteins were fractionated by hydroxyapatite chromatography, separated from nucleic acids by caesium chloride density gradient centrifugation and then subjected to two-dimensional gel electrophoresis. Subsequent autoradiography revealed that at least thirty protein species were modified. Both histones and non-histone proteins were modified but the specific activity of the latter group was greater. Proteins bearing different amounts of ADPR were resolved by this method, the average chain length of the non-histone protein fraction being greater than the histone fraction. In fact, in the case of one protein species there was evidence for the existence of several forms with different numbers of ADPR residues.

Lichtenwalner & Suhadolnik (1979) examined the nature of nuclear proteins modified by the addition of ADPR or 2'dADPR by either NAD^+ or 2'd NAD^+ in nuclei isolated from HeLa cells and rat liver. In nuclei labelled with NAD^+ the ADPR moiety was preferentially transferred to the H1 histones. However, in nuclei labelled with 2'd NAD^+ the 2'dADPR moiety was preferentially transferred to the non-histone proteins. In addition, the chain length of ADP-ribosylated histones and non-histone proteins was shorter with 2'd NAD^+ than with NAD^+ . Both these phenomena demonstrate the importance of the 2'hydroxyl group of NAD^+ in the ADP-ribosylation of nuclear proteins.

An alternative method for determination of protein modification has been to analyse the ADP-ribosylation of specific proteins in a reconstituted system of purified or partially purified poly(ADPR) synthetase, DNA and radiolabelled NAD^+ . In this way, Yoshihara et al. (1975) demonstrated the ADP-ribosylation of a $\text{Ca}^{2+}, \text{Mg}^{2+}$ -dependent endonuclease and Okazaki et al. (1980a,b) demonstrated the ADP-ribosylation of all the individual histone fractions. Yoshihara et al. (1977) showed that poly(ADPR) synthetase itself acted as an acceptor. Jump & Smulson (1980) showed that poly(ADPR) synthetase and a major non-histone protein acceptor of poly(ADPR) in HeLa cell nuclei, protein C, co-purified and thus substantiated the results of Yoshihara et al. (1977). The enzyme and acceptor protein had the same molecular characteristics i.e. $112,000\text{M}_r$ and 5.2S. Furthermore, analysis of the turnover of poly(ADPR) on both species, by pulse-chase studies, revealed a similar turnover rate. The existence of an endogenous acceptor of purified poly(ADPR) synthetase has also been reported (Caplan et al., 1979).

Okayama et al. (1978) developed a method which was specific for the isolation of ADP-ribosylated proteins from unmodified proteins. The method exploited the cis-2',3'diol of the 'nicotinamide' ribose of ADPR which forms a complex with immobilized aminophenylboronic acid. Using this dihydroxyboryl polyacrylamide column they showed that histones H1, H2B, H2A, the HMG proteins (Hayaishi et al., 1979; Okayama et al., 1978), A24 protein (Okayama & Hayaishi, 1978), and several non-histone proteins (Okayama et al., 1978) were modified. The chain length of

ADPR on these proteins appeared to be less than 2.

Adamietz et al. (1979, 1980) used a similar technique, employing boronate cellulose column chromatography, to isolate (ADPR)_n-protein conjugates from EAT cell nuclei. Since the ADP-ribosylated material had a great tendency to aggregate, high concentrations of guanidium chloride, urea or SDS were employed throughout. SDS gel electrophoresis revealed numerous proteins which coincided with [³H]ADPR (detected by fluorography) of mean chain length of 20. Isolation of histones from non-histone proteins by cation-exchange chromatography revealed that only a small proportion of both label and protein was present in this fraction. However, after specific detachment of ADPR from the proteins of both fractions, followed by SDS gel electrophoresis, histones banded at the positions of the unmodified species and a large proportion of the non-histone protein bands moved down the gel. Thus, ADP-ribosylation dramatically changed the physico-chemical properties of the proteins, especially those of histones H2A and H4 which were actually shifted to the non-histone protein portion of the gel on modification. The bulk of (ADPR) acceptors were non-histone proteins, in particular four proteins of molecular weight ranging from 12,000 to 125,000. Modified histone H1 comprised only a very small percentage of ADP-ribosylated protein. An interesting difference observed between these last two studies is that, although ADP-ribosylated proteins are isolated on much the same basis, the average chain lengths reported differ substantially. This may be accounted for to some extent

by the different temperatures and NAD^+ concentrations used by the two groups. The significance of the NAD^+ concentration in vivo must be borne in mind when studying protein ADP-ribosylation in vitro. Furthermore, the temperature employed for analysis of ADP-ribosylation in vitro is the optimum for ADP-ribosylation; such conditions do not exist in vivo. A further criticism of this method is that the pH value employed during chromatography, pH 8.2, has been shown to permit the release of a significant amount of ADPR from protein, particularly from HMG proteins 1 and 2 (P. Stone, personal communication).

All the above work has been involved with the study of nuclear proteins ADP-ribosylated in vitro and demonstrates that the ADP-ribosylation of these proteins appears to be very heterogeneous. Some of this heterogeneity may be a reflection of the different conditions used during the incubation of nuclei with radiolabelled NAD^+ or the different methods used to isolate the resultant ADP-ribosylated proteins. Only a relatively small number of studies have been carried out in vivo (Ueda et al., 1975; Young & Sweeney, 1978, 1979; Adamietz et al., 1978; Minaga et al., 1978). The results of these few studies already suggest that the ADP-ribosylation of nuclear proteins in vivo differs substantially from that in vitro in terms of chain length, protein-ADPR linkage and type of protein modified (see Section 1.6). It seems that the development of techniques for the isolation and analysis of proteins ADP-ribosylated in vivo is most pertinent to the elucidation of the function of this protein modification.

1.8 BIOLOGICAL FUNCTION OF ADP-RIBOSYLATION

Since the original discovery of poly(ADPR) and the finding that it is associated with chromatin, the polymer has been expected to play some regulatory role in nuclear function. The finding that ADPR of varying chain lengths is covalently bound to nuclear proteins supports this contention. However, the function of poly(ADPR) and the ADP-ribosylation of nuclear proteins is still not known. The association of poly(ADPR) synthetase with chromatin and its dependency on DNA, though, does indicate a close relationship between poly(ADP-ribosyl)ation and chromatin activities. However, most of the information available is circumstantial and fragmentary and even contradictory results have been reported.

Early approaches to determine the function of this protein modification involved isolating nuclei from cells in various states and attempting to correlate poly(ADPR) synthetase activity with a particular cell state. The validity of such in vitro measurements, though, must be treated with caution since the state of the chromatin itself in isolated nuclei will be altered significantly from that in intact cells by the assay conditions employed. Analyses of ADP-ribosylated protein, in an attempt to correlate type or amount of protein modified with a particular function, have also proved difficult because of the isolation techniques available. These and other problems have contributed to the difficulty in determining the precise role of ADP-ribosylation. It may be that, rather than being involved with a particular

cell function, ADP-ribosylation is involved with a factor common to all of these, such as accessibility to various regions of DNA involved in e.g. DNA replication, repair or transcription. The recent development of permeabilized cell systems, sensitive radioimmunoassays and techniques for the isolation and analysis of proteins ADP-ribosylated in vivo might provide a more realistic indication of the precise function of this protein modification. Therefore, bearing in mind the pitfalls of various experimental approaches, the possible functions of poly(ADPR) are described below.

1.8.1 DNA Replication

The association of poly(ADPR) synthetase with chromatin (Ueda et al., 1968), its dependency on DNA (Yoshihara, 1972), stimulation by histones (Yamada & Sugimura, 1973) and inhibition by thymidine (Preiss et al., 1971) may suggest that poly(ADPR) is involved in some aspect of DNA replication. The inhibitory effect of DNA on poly(ADPR) glycohydrolase (Hilz et al., 1974; Hayaishi & Ueda, 1974) supports this contention.

Burzio & Koide (1970) reported that when nuclei of rat liver were preincubated with NAD^+ a dose-dependent inhibition of $[^3\text{H}]\text{dTTP}$ incorporation into DNA was observed on formation of poly(ADPR). Nicotinamide added to the incubation medium prevented this inhibition. Addition of DNA polymerase to the assay system did not alter the incorporation of $[^3\text{H}]\text{dTTP}$ but on addition of calf thymus DNA incorporation was restored. This suggested that

inhibition was due to a suppression of the template capacity for DNA synthesis rather than inhibition of DNA polymerase. The same phenomenon was observed in nuclei of Physarum polycephalum (Brightwell & Shall, 1971), regenerating liver (Burzio & Koide, 1972), Ehrlich carcinoma and HeLa S3 cells (Hilz & Kittler, 1971). Hilz & Kittler (1971) expressed doubt as to the validity of the explanation provided since the inhibition of DNA polymerase activity in Ehrlich carcinoma and liver nuclei remained the same when exogenous DNA was added as template. Subsequently, Burzio & Koide (1971) showed that DNA obtained after complete removal of proteins from NAD^+ -treated rat liver chromatin possessed template capacity equivalent to that obtained from untreated chromatin; it seemed that inhibition was a result of ADP-ribosylation of chromatin-associated proteins. Furthermore, it was shown that the observed inhibition of DNA polymerase activity was due to the liberation of the enzyme from chromatin on poly(ADPR) formation (Yoshihara & Koide, 1973). Nagao et al. (1972) however, suggested that inactivation of the DNA polymerase itself occurred since activity of total or solubilized enzyme remained depressed. The ADP-ribosylation of polymerase was not demonstrated though.

In 1973 Burzio & Koide demonstrated an indirect inhibition of DNA polymerase activity caused by an effect on the DNA template itself. In the presence of NAD^+ and therefore poly(ADPR) synthesis Ca^{2+} , Mg^{2+} - dependent endonucleolytic activation of DNA, by formation of 3'OH primer groups, was prevented. Subsequently, this was

shown to be a result of direct ADP-ribosylation of a Ca^{2+} , Mg^{2+} - endonuclease (Yoshihara et al., 1974, 1975).

Inhibition was dependent on bound monomers or small oligomers of ADPR, but not polymers.

No inhibition of DNA synthesis by ADP-ribosylation could be observed in Novikoff hepatoma (Burzio & Koide, 1972), lymphocytes (Lehmann & Shall, 1972) or leucocytes (Burzio et al., 1975). The results of Lehmann & Shall were more physiologically significant since they used a DNA polymerase assay which approached conditions existing in vivo.

In contrast to any of the above results Roberts et al. (1973, 1974) showed a significant enhancement of DNA polymerase activity as a result of ADP-ribosylation in HeLa cells. The stimulation was specific for formation of poly(ADPR) and varied through the cell cycle. The release of template restriction was proportioned to the capacity of a given cell to synthesize poly(ADPR). A concentration of 1mM NAD^+ was sufficient to maximally stimulate DNA synthesis which was caused by exposure or generation of additional 3'OH primer sites. The ability of poly(ADPR) to stimulate DNA synthesis was found to be inversely proportional to template restriction through the growth cycle of HeLa cells.

More recently, Janakidevi (1978) has shown that removal of lysine-rich histones or treatment with heparin increases DNA synthesis in isolated nuclei from pig aorta. It was concluded that the removal of lysine-rich histones and poly(ADPR) synthetase (which are co-extracted) exposes initiation sites for DNA synthesis. Tanigawa et al. (1978a)

showed that preincubation of isolated nuclei from chick-embryo or hen liver with NAD^+ increased and decreased dTTP incorporation into DNA, respectively. Reconstitution experiments revealed that the factors responsible for stimulation or suppression were present in the 0.35M NaCl extract of liver nuclei. The stimulation of DNA synthesis observed in chick-embryo liver nuclei was later shown to be due to increased accessibility of the DNA to nuclease (Tanigawa et al., 1978b). Subsequently, it was shown (Kitamura et al., 1979) that administration of glucocorticoid hormone to chick-embryos caused a decrease in both DNA and poly(ADPR) synthesis.

An interesting observation was made by Ghani & Hollenberg (1978a) with respect to cell division, O_2 levels and poly(ADPR) synthesis. It had previously been observed that lowering ambient O_2 increased cell division (Hollenberg, 1971). Using chick-embryo heart cells Ghani & Hollenberg, (1978a) demonstrated that nuclei exhibited higher poly(ADPR) synthetase activity from cells grown in 20% (v/v) O_2 than 5% (v/v) O_2 . They also showed an increased amount of poly(ADPR) in vivo in cells grown in 20% (v/v) O_2 . It was suggested that in rapidly dividing cells (those grown in 5% (v/v) O_2) the redox potential shifts $\text{NAD}^+ \rightarrow \text{NADH}$, thus reducing poly(ADPR) synthesis and increasing DNA synthesis (Ghani & Hollenberg, 1978b).

However, nearly all the above analyses are a result of in vitro experimentation and as such probably do not reflect physiological ADP-ribosylation or semi-conservative DNA replication; the endogenous incorporation of

deoxyribonucleotides by isolated nuclei is a poor index of in vivo DNA replication. Also, the evidence for the direct involvement of DNA polymerase or Ca^{2+} , Mg^{2+} -dependent endonuclease in DNA synthesis and cell proliferation is by no means unambiguous.

In an attempt to overcome some of these problems Berger et al. (1978a) have suggested that permeabilized cells are better models for studying ADP-ribosylation and its relation to DNA synthesis than isolated nuclei. Cells are permeabilized by a fifteen minute hypotonic cold shock treatment and consequently become permeable to exogenously supplied nucleotides. In such a system DNA synthesis is semiconservative (Berger & Johnson, 1976) and occurs as extensions of replication sites that were active in vivo i.e. as elongation (Berger et al., 1977). In addition, the length and extent of synthesis is greater than that produced in isolated nuclei and the products are of higher molecular weight (Berger et al., 1977). In summary, the system closely reflects that in vivo. Permeabilized cells were also shown to be capable of forming poly(ADPR) from exogenously added $[\text{}^3\text{H}]\text{NAD}^+$; labelled, acid-insoluble material formed was resistant to DNase and RNase but more than 95% was degraded by snake venom phosphodiesterase (Berger et al., 1978a).

Using this system it was shown that inhibition of poly(ADPR) synthetase had no effect on DNA synthesis and that inhibition of DNA synthesis had no effect on poly(ADPR) synthesis in L cells. It was also demonstrated that when simultaneous synthesis of both polynucleotides occurred,

the synthesis of one did not affect the other. Furthermore, the rate of DNA synthesis was highest in the exponential phase of the growth cycle whereas that of poly(ADPR) synthesis was greatest in the stationary phase. However, if the activity of poly(ADPR) synthetase was measured in the presence of DNase I and Triton X-100 it did not vary in cells from log or stationary phase cultures but exhibited a maximal activity. This indicates that the physiological activity of the enzyme varies, possibly as a result of differences in availability of endogenous acceptors, while the total amount of enzyme remains constant. In addition, they showed that decreased DNA synthesis in cells subjected to acute glucose deficiency, vaccinia virus infection or cytosine arabinoside treatment always resulted in increased poly(ADPR) synthetase activity (Berger et al., 1978b).

All these studies showed that the greatest increase in poly(ADPR) synthesis occurred on cessation of DNA synthesis. However, a transient increase in poly(ADPR) synthesis is seen in cells actively synthesizing DNA, in agreement with the results of Lehmann et al. (1974). The relationship between DNA and poly(ADPR) synthesis may also be related to the observation by Kidwell & Mage (1976) that poly(ADPR) synthesis in synchronized cells occurred mainly as cells passed through S to G2 phase.

Berger et al. (1978c) also showed that differences occurred in normal and chronic lymphocytic leukemia (CLL) lymphocytes when subjected to mitogenic stimulation by phytohaemagglutinin (PHA). In both types of lymphocyte the stimulation caused an increase in ADP-ribosylation.

The normal cells also showed the expected increase in DNA synthesis on stimulation but in CLL cells this response was decreased and much delayed. It was suggested that this may be due to DNA damage. These results are the opposite to those in L cells with respect to the relationship between DNA and poly(ADPR) synthesis. Thus, although permeabilized cell systems have proved very useful for the analysis of poly(ADPR) and its relation to nuclear function, the complexity of the intact cell does not allow at this present moment in time a direct correlation between the synthesis of DNA and poly(ADPR).

1.8.2 Cell Proliferation

Pyridine nucleotide levels are low in tumours and in tissues undergoing rapid cellular proliferation (Morton, 1961). In addition, a significantly higher activity of poly(ADPR) synthetase has been noted on many occasions in the nuclei of dividing versus resting cells: hepatomas versus normal liver cells (Hilz et al., 1974), regenerating versus resting liver (Leiber et al., 1973), mitogen-stimulated versus unstimulated lymphocytes (Lehmann et al., 1974), leukemic versus normal leukocytes (Burzio et al., 1975), hormone-stimulated versus unstimulated oviducts (Müller et al., 1974), SV40-transformed cells versus untransformed cells (Miwa et al., 1976), and in serum-stimulated versus normal fibroblasts (Furneaux & Pearson, 1978). However, in neonatal versus adult rat liver (Leiber et al., 1973) and AH-130 hepatoma versus adult liver (Yamada et al., 1973) the activities of poly(ADPR)

synthetase were the same. Furthermore, no direct correlation between poly(ADPR) synthesis activity and DNA content or synthesis could be observed in a number of other cells (Leiber et al., 1973; Hilz et al., 1974; Lehmann et al., 1974). It seems that the only case when poly(ADPR) synthetase activity was always found to increase was on transition of cells or tissues from the non-growing status to the growing status, as on partial hepatectomy (Leiber et al., 1973) PHA - (Lehmann et al., 1974) serum - (Furneaux & Pearson, 1978) or oestrogen - (Müller, 1974) stimulation.

Stone & Shall (1975) showed that the specific activity of poly(ADPR) synthetase in isolated nuclei of cultured mouse fibroblast (LS) cells varied through the growth cycle. The activity of the enzyme increased approximately three-fold during the logarithmic phase of the growth cycle and was correlated with increase in cell number and therefore cell proliferation. However, poly(ADPR) synthetase activity was highest when DNA synthesis was lowest. The more recent results of Berger et al. (1978a), using a permeabilized cell system, agree with these results. This indicates that if poly(ADPR) synthetase activity is correlated with cell proliferation then it is involved in some process other than DNA synthesis.

In an attempt to correlate endogenous levels of mono(ADPR) and poly(ADPR) in tissues with different proliferation rates, Adamietz et al. (1974) analysed the ratio of NH_2OH -sensitive oligo(ADPR) to mono(ADPR) residues in

proliferating versus non-proliferating tissues. On quantitation it was found that, although total and NH_2OH -sensitive oligo(ADPR) residues did not differ in both types of tissue, significantly lower levels of protein-bound, NH_2OH -sensitive mono(ADPR) residues were found in proliferating versus non-proliferating tissues. This situation was also found in vivo (Stone & Hilz, 1975). Using permeabilized EAT cells these results were substantiated by the finding that poly(ADPR) synthetase activity was closely related to changes in endogenous mono(ADPR)-protein conjugate levels during progression from the proliferating to the non-proliferating state; an increase in both poly(ADPR) synthetase and NH_2OH -resistant mono(ADP-ribosyl)ated proteins was observed (Bredehorst et al., 1979).

Inhibitors of poly(ADPR) synthetase have been shown to cause a reduction in cell proliferation. Using a clone of HeLa cells resistant to 5-methylnicotinamide Kidwell & Burdette (1974) showed that, although the growth of cells sensitive to 5-methylnicotinamide was completely arrested, the resistant cells were able to grow at near normal rates. Müller et al. (1978) showed that formycin B inhibited poly(ADPR) synthetase and cell proliferation.

Thus, although no direct correlation between poly(ADPR) synthetase activity and cell proliferation can be observed at the present time it may be that the development of, for example, methods for determination of levels and type of ADP-ribosylated protein in vivo will end in the elucidation of such a correlation.

1.8.3 DNA Transcription

The first report suggesting a possible role for poly(ADPR) in DNA transcription was that of Haines et al. (1969) who observed that rat liver cells engaged in RNA synthesis possessed a higher poly(ADPR) synthetase activity than cells engaged in DNA synthesis.

In an attempt to clarify whether poly(ADPR) has a direct effect on RNA synthesis various workers have tried to correlate poly(ADPR) synthetase activity with RNA synthesizing capacity of various cells. Hilz & Kittler (1971) showed that there was no change in poly(ADPR) synthetase activity in livers of sham-operated, adrenalectomized or cortisol-treated rats, suggesting no role in DNA transcription. In contrast, Müller et al. (1974) showed that on induction of gene expression in quail oviducts by addition of oestrogen, an increase in RNA polymerase I and II activities is accompanied by a strong decrease in poly(ADPR) synthetase activity. However, this correlation may not hold since no corrections for possible changes in precursor pools were made. More recently, Tsopanakis et al. (1978b) observed that the specific activity of poly(ADPR) synthetase in nuclei and nucleoli of Tetrahymena is the same even though nucleoli exhibit high ribosomal transcriptional activity. Also, the activity of poly(ADPR) synthetase was the same in nuclei or nucleoli in the presence or absence of RNA synthesis.

An alternative approach has been to study the effect of poly(ADPR) formation on RNA synthesis. Burzio & Koide (1971) showed that formation of poly(ADPR) in isolated

liver nuclei did not change the capacity to synthesize RNA. However, Müller et al. (1974) have shown that on preincubation of quail oviduct nuclei with NAD^+ , the activity of RNA polymerase I decreased, possibly by ADP-ribosylation of the enzyme itself. Furthermore, the amount of ADPR associated with the polymerase was greater on reduced transcription (Müller & Zahn, 1976). More recent results of Furneaux & Pearson (1978) support the contention that inhibition of RNA polymerase I is by ADP-ribosylation of the enzyme itself. Momii & Koide (1980) showed that although the cessation of rRNA synthesis and decrease in activity of RNA polymerase occurred with maturation during spermatogenesis of mouse testis, poly(ADPR) synthetase activity increased. They suggested that a reciprocal relationship of ADP-ribosylating activity to the template activity for rRNA synthesis existed.

The contradictory results obtained above may be a result of in vitro analysis; studies in isolated nuclei may not necessarily reflect the situation in vivo because of the complexity of the processes being studied.

Mullins et al. (1977) approached the problem in a different way by attempting to isolate transcriptionally active and inactive regions of chromatin and correlating these with poly(ADPR) synthetase activity. By fractionating sonicated HeLa cell chromatin by ECTAM-cellulose chromatography and glycerol gradient centrifugation, they concluded that the synthetase activity was primarily associated with transcriptionally active chromatin. However, Yukioka et al. (1978) pointed out that mechanical

shearing, as used by Mullins et al. (1977), did not adequately separate active and inactive regions of chromatin and also that sonication drastically altered chromatin structure. Thus, Yukioka et al. (1978) used the milder and more sensitive technique of Gottesfeld et al. (1974, 1975) to separate chromatin into transcriptionally active and inactive fractions and subsequently showed that poly(ADPR) synthetase is not preferentially located in transcriptionally active regions of chromatin.

An indirect indication of involvement of poly(ADPR) in DNA transcription is the finding that protein A24, a repressor of ribosomal gene activity (Ballal & Busch, 1973; Ballal et al., 1974), is ADP-ribosylated (Okayama & Hayaishi, 1978). Whether this protein is actually ADP-ribosylated in vivo, however, remains to be seen on the development of techniques for the isolation and analysis of proteins ADP-ribosylated in vivo.

1.8.4 Cell Cycle

The possibility that ADP-ribosylation may be involved in events of the cell cycle, such as DNA synthesis or the actual timing of cell cycle events, has been studied by a number of groups by determining enzyme activity during the cycle in isolated nuclei from synchronised cells. Although no consistent picture could be observed, most results suggested that poly(ADPR) synthetase activity was highest in G2 or G1 phases and lowest in S phase (see Hilz & Stone, 1976). In all of these studies synchrony was obtained by use of different metabolic inhibitors. However, many

agents which arrest cell growth have drastic effects on poly(ADPR) synthesis (Berger et al., 1978a,b). In fact, the use of thymidine to obtain synchrony may affect poly(ADPR) synthetase itself in that it is an inhibitor of the enzyme (Preiss et al., 1971). In addition, levels of poly(ADPR) may be elevated because of DNA damage incurred during the nuclear isolation procedures employed. Thus, the differences obtained between these studies may be due to the imbalance in cell growth and the processes associated with it. The unsuitability of isolated nuclei for elucidating the role of ADP-ribosylation in the cell cycle is also demonstrated in the more recent experiments of Tanuma et al. (1978).

Kidwell & Mage (1976) tried to obtain a more realistic estimation of changes in ADP-ribosylation during the cell cycle by using a radioimmunoassay to detect levels of poly(ADPR) in HeLa cells. They observed a six-fold increase in poly(ADPR) as cells traversed from early to late S phase, followed by a rapid decrease then a ten-fold increase at the S-G2 transition point. Levels correlated with poly(ADPR) synthetase activity. However, since the chain length of poly(ADPR) may vary through the cell cycle, more information on the specificity of the antibody is required; long chain polymer may compete much more effectively for antibody binding than oligo(ADPR) or mono(ADPR).

Using a permeabilized cell system Berger et al. (1978b) showed that after treatment of L1210 cells with cytosine arabinoside, cells accumulated in G2 phase and exhibited

a resultant increased poly(ADPR) synthetase activity. Rastl & Swetly (1978) noticed a similar effect when erythroleukemic mouse spleen cells were arrested in G1 phase. Some of the increases in poly(ADPR) synthetase activity may therefore be accounted for, in part, by the increase observed on cells reaching a resting state after proliferation as previously described.

Thus, in an attempt to approach an in vivo situation Berger et al. (1978d) used a combination of cell permeabilization and mitotic selection, shown to give a high degree of synchrony of essentially unperturbed cells (Terasima & Tolmach, 1963), to study synthesis of poly(ADPR) in Chinese hamster ovary (CHO) cells. They showed that the level of poly(ADPR) synthesis was elevated during G1, fell to its lowest during S phase, then rose in G2 through G1 phases. The DNase-responsive i.e. total potential poly(ADPR) synthetase activity was relatively constant through the cell cycle but showed a small peak towards the end of S phase. They suggested that this may be due to the synthesis of the enzyme protein and that the level of enzyme is restored to its usual value as the cells subsequently divide. This observation may account for the maxima observed during S phase by other workers (Roberts et al., 1973; Colyer et al., 1973; Kidwell & Mage, 1976). Also, DNA damage during isolated nuclei may be equivalent to the DNase-responsive system in that total or near total poly(ADPR) synthetase activities are expressed.

Wielckens et al. (1979) exploited the natural synchrony

of the multinuclear plasmodia of the slime mould Physarum polycephalum to study levels of protein-bound mono(ADPR) during the cell cycle. Quantitation, using a sensitive radioimmunoassay, showed that total mono(ADPR) levels decreased from mitosis through S phase and then increased through G2 phase to M phase. However, on analysis, different patterns of hydroxylamine-sensitive and hydroxylamine-resistant mono(ADPR)-protein conjugates were observed. Hydroxylamine-resistant residues increased during S phase, remained constant at the S/G2 phase boundary and then decreased at mid-G2 phase. Hydroxylamine-sensitive residues increased at the S/G2 phase boundary, then decreased at mid-G2 phase, followed by a sharp increase and decrease before and after mitosis, respectively. These findings may indicate that nuclear ADP-ribosylation reactions serve more than one function.

Studies on cell cycle events and its relation to synthesis of mono(ADPR) or poly(ADPR) are therefore complicated not only by the processes that occur during the cycle but also by the heterogeneity of ADP-ribosylation. It appears that the permeabilized cell/mitotic selection system of Berger et al. (1978d) and the analysis of protein-bound ADPR by Wielckens et al. (1979) may best reflect the changes in ADP-ribosylation during the cell cycle at this present moment in time.

1.8.5 Cell Differentiation and Development

Since they had previously observed a correlation between pyridine nucleotide levels and cellular differentiation, Caplan & Rosenberg (1975) studied the differentiation

of mesodermal cells of chick-embryo limb buds into either muscle or cartilage to try to find any involvement of observed fluctuations in NAD^+ levels, and therefore poly(ADPR), in differentiation. It was shown that 3-acetylpyridine potentiated cartilage differentiation and that this was associated with a stimulation in rate of poly(ADPR) synthesis. It was proposed that the fluctuations in NAD^+ levels were 'sensed' and resulted in differential rates of ADP-ribosylation that were correlated with differentiation into either muscle or cartilage. However, this proposal was questioned by McLachlan et al. (1976) who showed that the effect of 3-acetylpyridine was mediated via a destruction of peripheral nerves and therefore affects growth rather than differentiation.

A differential effect in poly(ADPR) levels is also seen on hormone-induced differentiation in quail oviduct; poly(ADPR) synthetase activity increased on oestrogen treatment, concomitant with DNA and cell replication, and decreased on progesterone treatment and subsequent avidin synthesis (Müller et al., 1974).

Since these studies various reports have appeared suggesting a role for ADP-ribosylation in differentiation. One approach to this study has been to measure poly(ADPR) synthetase activity in differentiating cells. Burzio & Koide (1977) showed that during oocyte maturation in Xenopus a dramatic condensation of the chromosomes occurs and that just before this there is a three-fold increase in poly(ADPR) synthetase activity. Farzaneh & Pearson (1979) also showed a substantial increase in synthetase

activity during the embryonic development of Xenopus, thus substantiating the contention that a role for ADP-ribosylation in early differentiation may exist.

Young & Sweeney (1978) showed that maximum incorporation of [³H]adenosine into poly(A) and poly(ADPR) occurred at three to five hours after ovulation in the mouse ova, the time of normal fertilization. Poly(ADP-ribosyl)ation may therefore play a role in fertilization or in post-ovulation maturation. The same workers (Young & Sweeney, 1979) also showed that ADP-ribosylation occurred in the mouse one-cell embryo and that this differed in both average chain length and lability of the protein-ADPR bond compared to that in the unfertilized ovum. Such differential ADP-ribosylation may indicate that differentiation and poly(ADPR) synthesis are correlated in some way. Hilz et al. (1980) also analysed protein-bound ADPR levels in an attempt to correlate differentiation with poly(ADPR). By quantitating mono(ADPR) protein-bound residues from intact tissues at different stages of gene expression, by radioimmunoassay, they concluded that the state of differentiation may determine the endogenous levels of protein-ADPR conjugates. Porteus et al. (1979) exploited the ordered separation of intestinal epithelial cells into two related populations, one dividing (lower half of crypts) and one differentiating but not dividing (upper half of crypts and villi) to analyse the role of ADP-ribosylation in differentiation or cell division and DNA synthesis. The poly(ADPR) synthetase activity was found to be associated with those cells undergoing DNA

synthesis and mitotic division but was almost absent from those cells undergoing differentiation. In addition, the range of sizes of poly(ADPR) chains was greater in the dividing compared to the differentiating cells. Although this study does not preclude a role for ADP-ribosylation in cell differentiation it shows that ADP-ribosylation may be important in other processes as well. Caplan et al. (1979), using an assay for chemical quantities of poly(ADPR), showed that a three-fold decrease in poly(ADPR) per unit of DNA occurred during the early phases of chick limb mesenchymal cell development both in situ and in culture. However, one limitation of this assay was that it was not able to detect mono(ADPR) or small chain poly(ADPR). Whether these fluctuations in poly(ADPR) levels represent primary control elements or responses to control events involved in these processes is, at present, unclear.

Recently, using an indirect immunofluorescence technique, Ikai et al. (1980) showed that poly(ADPR) was synthesized in nuclei of lymphocytes and monocytes in normal blood but not in granulocytes or erythrocytes. However, poly(ADPR) synthesis was detected in myelocytes ('precursors' of granulocytes) in patients with acute myeloblastic leukemia as well as patients in blastic crisis of chronic myelocytic leukemia but not in normal blood or bone marrow. These results suggested that the capacity for synthesizing poly(ADPR) may serve as a marker of granulocyte differentiation.

An alternative approach to correlating poly(ADPR) synthetase activity and levels of ADP-ribosylation in

naturally differentiating cells has been to induce differentiation of cells. Yamada et al. (1978) showed that on treatment with poly(ADPR) mouse myeloid leukemia cells could be induced to differentiate, displaying phagocytic activity, Fc receptors and lysozyme activity. However, addition of dextran sulphate or poly(vinyl sulphate) was just as effective, so doubt is raised as to the physiological significance of this observation.

Rastl & Swetly (1978) using F4N Friend cells showed that, on induction of erythropoietic differentiation by butyrate, poly(ADPR) synthetase activity increased three-fold and this mainly affected the ADP-ribosylation of histones (Zlatanova & Swetly, 1980). Moroika et al. (1979), however, using 745 Friend cells showed that poly(ADPR) synthesis was suppressed on induction of differentiation by hexamethylenebisacetamide (HMBA) or dimethyl sulfoxide (DMSO) in the early exponential growth phase but with butyrate induction synthetase activity was transiently increased and then decreased. The effect of the inducers was not through a change in cell growth rate or poly(ADPR) degradation. The differences observed between these two groups may be attributed to either differences in cell line used - F4N is extremely sensitive to butyrate but relatively insensitive to HMBA (Moroika et al., 1979), or the difference in method of cell culture - Rastl & Swetly used fresh medium every day whereas Moroika et al. did not. The different effects of inducers of differentiation with respect to poly(ADPR) activity also complicates the analysis. This was substantiated by the effect of

nicotinamide, another inducer of differentiation, on HMBA, DMSO and butyrate induction; it enhanced the activity of the former two but inhibited that of the latter.

Terada et al. (1979) analysed the induction of erythroid differentiation in Friend cells by nicotinamide and related compounds. They found that all the compounds studied that had strong inhibitory effects on poly(ADPR) synthesis in vitro induced differentiation. This property is not a prerequisite of inducers, though, since N'-methyl-nicotinamide which did not inhibit the enzyme in vitro, was the most effective inducer of differentiation. Whether this reflects the situation in vivo is, as yet, unknown; N'-methylnicotinamide may inhibit synthetase activity under these conditions.

It is clear that ADP-ribosylation does have some connection with processes involved in differentiation. Whether it is involved in the direct control of such processes or is a result of some other aspect of control, and whether ADP-ribosylation is enhanced or suppressed at this time is, at present, unknown.

1.8.6 DNA Repair and Fragmentation

The observation by Roitt (1956) that alkylating agents and other treatments known to damage DNA result in depletion of cellular NAD⁺ levels, together with the more recent finding that poly(ADPR) synthetase is stimulated by fragmented or damaged DNA (Miller, 1975a), indicate the possible involvement of ADP-ribosylation in DNA repair mechanisms.

Miller (1975a) reported that the addition of DNase I or micrococcal nuclease to HeLa nuclei resulted in a four- to six-fold increase in poly(ADPR) synthetase activity. Analysis of the poly(ADPR) synthetase products in the presence or absence of DNase I showed only minor differences in chain length indicating that the main effect of the nuclease was through initiation of new chains of poly(ADPR) (Miller, 1975b). It was also postulated that the apparent discrepancy in the observed effect of DNase I on the synthetase reaction in previous investigations was due to incorrect assay conditions.

Since these observations were made a number of workers have studied the effect of damaging DNA on the synthesis of poly(ADPR). Whish et al. (1975) and Smulson et al. (1975) showed that the poly(ADPR) synthetase activity of nuclei of Physarum polycephalum and HeLa cells, respectively, was stimulated after pretreatment with streptozotocin, the 2-deoxy-D-glucose derivative of the alkylating agent, N-methyl-N-nitrosourea. Studies of the effect of alkylating agents on poly(ADPR) synthesis have been widespread. Among those agents shown to stimulate ADP-ribosylation are N-methyl-N-nitrosourea (MNU) (Smulson et al., 1977; Skidmore et al., 1979; Sudhakar et al., 1979a,b; Nduka et al., 1980) and N-methyl-N'-nitro-N-nitroguanidine (MNNG) (Berger et al., 1979a, 1980a,b; Jacobson et al., 1980). Jacobson (1978) analysed the NAD⁺ content of 3T3 cells and mitogen-stimulated human lymphocytes after exposure to N-alkyl-N-nitroso compounds that were direct-acting, indirect-acting or non-carcinogens. Direct-acting carcinogens caused large

decreases in NAD^+ in both 3T3 cells and lymphocytes, indirect-acting caused a decrease in lymphocytes but not 3T3 cells and non-carcinogens did not affect the NAD^+ content of either cell type.

Sudhakar et al. (1979a) showed that MNU increased the availability of protein acceptors for ADP-ribosylation. The same group (Sudhakar et al. (1979b) compared alkylation of chromatin by MNU and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) in HeLa cells; MNU primarily causes DNA strand breakage whereas CCNU causes cross-linking. In contrast to MNU, CCNU caused a slight decrease in poly(ADPR) synthetase activity. They showed, by nuclease digests, that this differential effect may be explained by the alkylation sites of the two compounds; MNU preferentially alkylates internucleosomal regions of DNA, suggested as the sites of synthetase binding (Mullins et al., 1977), whereas CCNU alkylates the core particles. Since MNU and CCNU may also carbamoylate proteins, the preferential modification of non-histone proteins by CCNU may have a direct effect on the synthetase itself. The increase in poly(ADPR) synthetase activity was apparently exerted through the initiation of new chains, in agreement with Miller (1975a). Davies et al. (1976, 1977) have shown that poly(ADPR) levels also increase on treatment of nuclei with the polypeptide antibiotic neocarzinostatin.

Other treatments known to damage DNA have also been shown to stimulate poly(ADPR) synthetase activity. Among these are x-irradiation (Benjamin & Gill, 1978, 1980), γ -irradiation (Skidmore et al., 1979) and uv-irradiation

(Berger et al., 1979a, 1980a,b). All these workers permeabilized cells by either mild detergent lysis (Benjamin & Gill) or cold hypotonic shock (Skidmore et al., Berger et al.) and, as such, analysed the response of poly(ADPR) synthesis to DNA damage under conditions which closely reflected those in vivo. Berger et al. (1979a) characterized the response in detail. They showed that cycloheximide did not inhibit the synthesis of poly(ADPR) and DNA on DNA damage and therefore that the response is carried out by pre-existing enzymes. This 'reserve' of poly(ADPR) was also demonstrated on DNase I treatment. Using alkaline CsCl gradients with bromodeoxyuridine triphosphate density labelling of DNA, they demonstrated that the unscheduled synthesis of DNA in response to uv-irradiation was a result of the repair mode of DNA synthesis, rather than DNA replication. Berger et al. (1980a) also exploited the inability of Xeroderma pigmentosum (XP) cells to repair uv-induced DNA damage to analyse the relationship between DNA repair and poly(ADPR) synthesis; XP cells exhibited an increase in poly(ADPR) synthesis in response to treatment with MNNG but not uv. Subsequently, they showed that treatment of uv-irradiated XP cells with M.luteus uv endonuclease restored the ability of XP cells to increase their poly(ADPR) synthesis on uv irradiation (Berger et al., 1980b) and, furthermore, that this increase in poly(ADPR) synthesis preceded unscheduled DNA synthesis (Berger & Sikorski, 1980b).

Benjamin & Gill (1980) proposed that two general types of nuclear ADP-ribosylation exist, one predominant in cells

with relatively undamaged DNA and associated with mono(ADP-ribosyl)ated or oligo(ADP-ribosyl)ated proteins and the second occurring after DNA damage and associated with poly(ADP-ribosyl)ated proteins. Juarez-Salinas et al. (1980) also showed, by quantitation, that a dramatic increase in poly(ADPR) levels occurred in response to DNA damage. An interesting observation by Benjamin & Gill (1980) was that fragments of DNA with flush ends were ten times more effective at stimulating poly(ADPR) synthesis than those with 2-4 nucleotides extending from the 5'PO₄ ends.

Inhibitors of poly(ADPR) synthesis have been used to further investigate the relationship between ADP-ribosylation and DNA repair. In fact, some of the inhibitors of poly(ADPR) synthesis, such as theophylline and caffeine, are also known inhibitors of DNA repair (Cleaver & Thomas, 1969; Lehmann & Kirk-Bell, 1972). Smulson et al. (1977) showed that mice injected with L1210 tumour cells exhibited an increased survival over controls in the presence of MNU and that co-administration of nicotinamide increased this survival to an even greater extent. Shall et al. (1977) and Nduka et al. (1980) substantiated this apparent enhancement of cytotoxicity of DNA-damaging reagents by inhibitors of poly(ADPR) synthetase and showed that the effect was synergistic. Using permeabilized mouse L1210 cells Durkacz et al. (1980) examined the relationship between poly(ADPR) synthetase activity, cell survival and excision repair in conjunction with inhibitors of the synthetase. It was shown that specific inhibitors prevent rejoining of DNA strand breaks caused by dimethyl sulphate (DMS) and, as a result, cytotoxicity was enhanced. Although all

inhibitors had been shown to be effective in this respect it could not be excluded that they might also be inhibiting some aspect of DNA repair directly. Therefore, as a control, cells were depleted of NAD^+ by growing in nicotinamide-free medium. Using alkaline sucrose gradients it was shown that the rejoining of strand breaks was also prevented under these conditions. The same phenomenon was demonstrated by Jacobson & Narasimhan (1979) using 3T3 cells.

All the above results suggest that ADP-ribosylation of nuclear proteins plays some, as yet unknown, role in DNA repair. The synthesis of poly(ADPR) in response to DNA damage may serve to modify chromatin structure so that enzymes of DNA repair can gain access to the damaged DNA (see Section 1.8.7). Many properties of the poly(ADPR) system suggest that it is well suited to a role in DNA repair because (i) it is a negatively charged polynucleotide, (ii) it is covalently linked to chromatin proteins, (iii) it is rapidly synthesized and degraded, (iv) the synthetase exists in large reserve quantities, and (v) the synthetase activity is both increased on DNA damage and is insensitive to inhibitors of protein synthesis.

Since poly(ADPR) synthesis increases in response to DNA damage and also on cessation of DNA synthesis (Berger *et al.*, 1978a,b,c) Berger *et al.* (1979b) carried out an analysis to determine whether DNA strand breaks might accompany suppression of DNA synthesis and, as such, account for the increased level of poly(ADPR) synthesis observed under these conditions. Rather than use

exogenous inhibitors, which may cause direct damage, to suppress DNA synthesis, they employed CHO cells in stationary phase of growth where density-dependent inhibition of DNA synthesis occurs. However, since DNA strand breaks might occur as a result of accumulation of toxic substances under these conditions, they also employed cs4-D3 cells as control. These are temperature-sensitive mutants of CHO cells in which DNA synthesis stops and cells arrest in the G1 phase of the cell cycle when cultures are shifted to 33°C. When both cell types were shifted to conditions to suppress DNA synthesis, poly(ADPR) synthesis was increased and DNA strand breaks appeared. In addition, both of these phenomena appeared to be characteristic of the G1 phase of the cell cycle. These workers subsequently pointed out that a number of other situations where poly(ADPR) synthesis is seen to be increased can be explained by cessation of DNA synthesis followed by DNA strand breakage. In all these cases cells accumulated in the G1 phase of the cell cycle, previously shown to exhibit the highest activity of poly(ADPR) synthetase (Berger et al., 1978c). For example, suppression of DNA synthesis is part of the differentiation process; increased levels of poly(ADPR) were observed in adult rat heart cells compared to neonatal rat heart cells (Claycomb, 1976) and also during induction of haemoglobin synthesis in murine erythroleukemia cells (Rastl & Swetly, 1978). More recently, Farzaneh et al. (1980) showed that in chick myoblasts the transition from the proliferating to the differentiating state was associated with DNA strand breakage and an increase in

poly(ADPR) synthetase activity. They also showed that although the expression of the differentiated state is inhibited by synthetase inhibitors, the appearance of DNA strand breaks was not, suggesting that the synthetase is involved in the process of differentiation at a point distal to the appearance of DNA strand breaks. These results are in agreement with those of Berger & Sikorski (1980b).

1.8.7 ADP-ribosylation and Chromatin Structure

It is well established that chromatin is a highly ordered structure whose structural integrity is dependent on a number of factors. Not least of these is the nucleosome, a 140 base-pair length of DNA wound round an octamer of core histones (H2A, H2B, H3 and H4) and linked to the next by a variable length of DNA associated with histone H1. The structure of chromatin may be altered at two levels: it may become extended during such processes as transcription or DNA repair, or the gross chromatin structure may be altered such as during chromatin condensation before mitosis. During these processes the association of various proteins with the DNA may alter substantially, as may the relationship between various regions of chromatin. One way in which some of these processes may be mediated is through the post-translational modification of proteins by, for example, ADP-ribosylation. ADP-ribosylation may be involved in either of the two levels of alteration in chromatin structure, although its low levels in vivo preclude the involvement of poly(ADPR)

as a major structural element.

Mullins et al. (1977) exploited the extremely tight association of poly(ADPR) synthetase with chromatin as a probe with which to assess not only the distribution of a chromatin-associated enzyme within the chromatin substructure, but also to try to aid in the elucidation of the physiological importance of the apparent highly ordered structure of proteins along the chromosomal DNA. The enzyme activity was shown not to coincide with the position of core particles on sucrose gradients, but was present in that part of the gradient which was enriched in monomers with linker regions. By employing a polyacrylamide gel technique which was capable of separating mononucleosomes with and without linker regions, they showed that dimers and monomers containing linker regions possessed poly(ADPR) synthetase activity and concluded that this was the location of the enzyme within the chromatin substructure (Giri et al., 1978b).

In an attempt to demonstrate the importance of the structural integrity of chromatin and its effect on ADP-ribosylation of nuclear proteins Giri et al. (1978a) made use of the development of a two-dimensional gel system which allowed identification of modified proteins not only from the nucleus as a whole, but also in nucleosomal fragments of chromatin generated by digestion with micrococcal nuclease. When HeLa nuclei were incubated with NAD^+ before nuclease digestion it was shown that ADP-ribosylated histones were preferentially associated with mono and dinucleosomes, whereas higher oligonucleosomes exhibited

more extensive modification of non-histone proteins. It was suggested that ADP-ribosylation of histones occurs in nucleosomes that are either more susceptible to nuclease or are rendered more susceptible by ADP-ribosylation. On analysis of proteins ADP-ribosylated in nuclei or in nucleosomes, it was shown that in the former histones H1 and H2B were major acceptors with histones H2A, H3, HMG proteins and M1-M4 proteins being modified to a lesser extent, whereas in the latter only histones H1 and H3 were modified and ADP-ribosylation of HMG proteins, M1 and M4 proteins was greatly enhanced. Thus, it appears that core histones are only modified in 'native' chromatin, therefore emphasizing the importance of the interaction of linker-associated poly(ADPR) synthetase with core particles. The significance of this work may also be extended to possible differences in in vitro and in vivo ADP-ribosylated proteins; isolation of nuclei may critically alter chromatin structure, including DNA strand breakage, from that in vivo.

Recent studies, reconstituting HeLa cell poly(ADPR) synthetase with purified oligonucleosomal chromatin, have confirmed that there are strict steric requirements between histones and the enzyme in chromatin for effective modification of histones; with increase in nucleosomal number less enzyme automodification and non-histone protein modification occurred and histone modification increased. This suggested that on increased intramolecular folding of oligonucleosomes histone modification was favoured (Jump et al., 1980a). Furthermore, using trypsin

which cleaves the NH₂ termini of nucleosomal histones, Jump et al. (1980b) showed that the site of histone modification was near the NH₂ terminal. Since these basic amino acid residues can potentially interact with DNA this modification may significantly influence chromatin structure. One criticism of all this work is that it is not certain whether the protein modification being studied is initiated in vitro or is an extension of in vivo ADP-ribosylation.

Initial efforts to associate poly(ADPR) synthetase activity with functional aspects of chromatin showed that the enzyme is enriched in extended chromatin (Mullins et al., 1977) but not necessarily in transcriptionally active chromatin (Yukioka et al., 1978). Jump et al. (1979) investigated this relationship further by determining the distribution of poly(ADPR) synthetase and its acceptors in chromatin from mid-S phase HeLa cells. Chromatin was selectively cleaved by micrococcal nuclease at sites active in DNA replication. Digestion resulted in the release of nucleosomes enriched in ADP-ribosylated proteins, poly(ADPR) synthetase activity and nascent DNA from the DNA replicating fork. The results show that poly(ADPR) synthetase was maximally active in extended regions of chromatin, contiguous to the DNA replicating fork and also demonstrate that poly(ADPR) synthetase and ADP-ribosylated proteins may play some role in the organization of chromatin.

Butt et al. (1978) using sucrose density gradients to generate chromatin with differing nucleosome repeat number,

demonstrated that the specific activity of poly(ADPR) synthetase rises to a maximum with chromatin of 8-10 nucleosomes in length and then falls and levels off with increasing nucleosomal number, suggesting that a defined structure within the chromatin may limit the reaction. In fact, Finch & Klug (1976) showed that nucleosomes can be organized into solenoids with approximately 6-9 nucleosomes per helical turn. In addition, Butt et al. (1979) and Stratling et al. (1978) have shown a preferential cleavage of chromatin by micrococcal nuclease at a periodicity of 8 and 16 nucleosomes. The high specific activity of poly(ADPR) synthetase in octanucleosomes is partly explained by the observation that 90% of radioactivity occurred on protein C which may be the synthetase itself. Butt et al. (1979) proposed that this enzyme might be bound at a periodicity of 8 nucleosomes or in the mid-region of a 16-nucleosome structure.

An observation, which may bear some relation to the importance of histone H1 in maintaining higher-ordered chromatin structure (Finch & Klug, 1976; Thomas & Koller, 1977), was made by Stone et al. (1977). They showed that a dimer complex, consisting of two molecules of H1 in association with a single chain of poly(ADPR) 15 units in length, was synthesized in HeLa cell nuclei and postulated that it may be involved in cross-linking of H1 molecules on non-adjacent regions of chromatin. Subsequently, Byrne et al. (1978) showed that a correlation between induced chromatin condensation and the extent of histone H1-polymer complex synthesis existed. The reversibility of the

process was demonstrated by the observation that histone H1-polymer complex accumulation is inversely related to the glycohydrolase activity present (Lorimer et al., 1977). If the complex does play a role in cross-linking then the H1 in the complex must have a similar affinity for DNA as unmodified H1. Stone et al. (1978), using DNA-cellulose chromatography, showed that indeed modified and unmodified H1 interacted with DNA in a very similar fashion. In addition, they showed that the complex was degraded by glycohydrolase at a rate one ninth that of free poly(ADPR), indicating that the polymer is in a protected environment within the complex. Smulson et al. (1980) reported that synthesis of the complex occurs in large (16 nucleosomes) polynucleosomal structures, supporting a possible role in cross-linking of chromatin. However, the complex has not yet been detected in vivo (Adamietz et al., 1978a).

Janakedevi & States (1980) analysed the effect of ADP-ribosylation on chromatin by examining the melting profiles of solubilized chromatin pre-incubated with or without NAD⁺. It was demonstrated that ADP-ribosylation stabilizes chromatin as seen by an upward shift in the temperature required to melt polynucleosomes.

Kidwell et al. (1980) showed that the amount of ADP-ribosylation may be related to the physical state of the chromatin rather than variations in the polymer synthesizing or degrading enzymes. α -picolinic acid treatment of HeLa cells results in an accumulation of cells in G2 phase and an elevation of polymer levels. Analysis of the activity of poly(ADPR) synthetase and

glycohydrolase indicated that the elevated levels of polymer were due to a change of synthetase from an inactive to an active state i.e. an enhancement of exposure of synthetase and/or titrating out of glycohydrolase from the nuclear sap. Therefore, changes in polymer levels are a consequence of changes in chromatin structure. This does not preclude the possibility that the resultant ADP-ribosylated proteins may then lead to further changes in chromatin structure.

Polyamines have been shown to stimulate ADP-ribosylation in isolated nuclei from various tissues (Müller & Zahn, 1976; Tanigawa et al., 1977; Perrella & Lea, 1978; Whitby et al., 1979). Greatest stimulation is observed by spermine, then spermidine and least of all by putrescine. Tanigawa et al., (1977) showed that non-histone proteins were predominantly modified in the presence of spermine, whereas histones were predominantly modified in the presence of Mg^{2+} . Perrella & Lea (1978) showed that spermine caused increased ADP-ribosylation of histone H1 and non-histone proteins (mainly the acidic fraction) with concomitant decrease in ADP-ribosylation of core histones. The results of Whitby et al. (1979), using wheat nuclei, differed somewhat from the above in that polyamines as well as Mg^{2+} increased ADP-ribosylation of total histones and, furthermore, the distribution of ADPR amongst histone H1 and the core histones did not alter on stimulation. These results may be explained by differences between plant and animal nuclei. Whitby et al. (1979) also showed that the increase in ADP-ribosylation was not due to an inhibition of poly(ADPR) glycohydrolase

by polyamines. An increase in ADP-ribosylation of histone H1-poly(ADPR) complex was also observed on addition of polyamines or Mg^{2+} (Byrne et al., 1978). These workers showed, by means of light scattering techniques, that on addition of polyamines or Mg^{2+} an instantaneous aggregation of chromatin occurred and that this was concomitant with a stimulation in complex formation.

Perrella & Lea (1978) suggested that, since spermine and Mg^{2+} stimulate ADP-ribosylation to the same extent as Mg^{2+} alone, high Mg^{2+} concentrations mask the effect of spermine on ADP-ribosylation. Thus, a dynamic equilibrium may exist between free and bound polyamines which is dependent upon the concentration of divalent cations. Tanigawa et al. (1980) suggested, on the basis of binding experiments, that the sites of ADP-ribosylation on polyamine and Mg^{2+} stimulation are the same; an additive effect by spermine could only be seen in the presence of low concentrations of Mg^{2+} . They went on to show that the stimulation of ADP-ribosylation was a result of bound spermine (to nucleic acid) and is probably caused by alteration of chromatin conformation. Increased ADP-ribosylation was due to an increase in both chain length and chain number and the major ADP-ribosylated proteins were two non-histone proteins of M_r 130,000 and 70,000. In addition, nuclei ADP-ribosylated in the presence of spermine showed a seven-fold increase in $[^3H]$ dTMP incorporation into the acid-insoluble fraction. These results indicate that DNA synthesis in growing tissues containing polyamines at high levels, as is the case with tumours and the foetus, is

stimulated by polyamine-mediated ADP-ribosylation of nuclear proteins. This is likely to be a result of altered chromatin substructure and thus increased accessibility of enzymes to DNA.

An intriguing observation was made by Minaga et al. (1978) who showed that inhibitors of poly(ADPR) synthetase induce ornithine decarboxylase and as such the enzyme may be regulated by ADP-ribosylation. However, the product of ornithine decarboxylase, spermine, stimulates ADP-ribosylation in isolated nuclei, as described above. The significance of this finding has yet to be elucidated.

1.9 CONCLUSION

ADP-ribosylation of nuclear proteins appears to be a complex process. The activity of poly(ADPR) synthetase, levels of protein-ADPR conjugates, linkage of protein to ADPR and the type of protein modified all vary under, not only induced or natural conditions, but also according to the method or methods chosen to analyse the response. ADP-ribosylation appears to be involved in some way with a number of nuclear functions. However, attempts to correlate the modification with a particular cellular process have proved unsuccessful; it may be that ADP-ribosylation is involved in some factor common to all of these, such as accessibility to DNA. One aspect of the nucleus which does seem to have a clear-cut correlation with ADP-ribosylation is the fragmentation of DNA. Furthermore, many of the processes which have been observed to result in increased levels of poly(ADPR) are

associated with DNA fragmentation. DNA fragmentation may be involved in the accessibility of enzymes to DNA. However, this correlation must remain tentative until further results are obtained.

From preliminary results it is apparent that ADP-ribosylation in vivo may differ substantially from that in vitro. This is hardly surprising since the conditions used for isolation of nuclei may alter the structure of chromatin significantly from that in vivo. As an intrinsic part of the chromatin poly(ADPR) synthetase and glycohydrolase activities are likely to alter as a result of these perturbations in chromatin structure. Such changes in chromatin structure may, in fact, mimic those in vivo, resulting in stimulation or inhibition of poly(ADPR) synthetase or glycohydrolase activities. It is apparent that, to reveal the true function(s) of this modification, efforts must be made to analyse the system under in vivo conditions. The recent development of sensitive radio-immunoassays and permeabilized cell techniques, in addition to techniques for the isolation and characterization of some proteins ADP-ribosylated in vivo and the development of specific inhibitors of the synthetase, should aid considerably in achieving this goal.

Since ADP-ribosylation processes do differ in vitro and in vivo, the aim of this work was to develop a method for the quantitative and non-selective isolation of proteins ADP-ribosylated in vivo. The characterization of these modified proteins and the effect of various reagents on in vivo ADP-ribosylation may then provide valuable

information on the true function(s) of this modification.

The development of this method was made using mouse L1210 cells. ADP-ribosylated proteins from these cells were isolated and characterized. Chain length, susceptibility of the protein-ADPR linkage to various reagents, and number and type of protein modified were determined. The effect of various inhibitors of poly(ADPR) synthetase, and dimethyl sulphate, on the quantity and quality of ADP-ribosylation of protein in vivo was then observed, in an attempt to reveal some of the functional aspects of the modification in vivo. It was hoped that this preliminary analysis of ADP-ribosylation in vivo would reveal any differences between the process in vivo and in vitro and so provide a comprehensive basis for future experiments.

CHAPTER 2

M A T E R I A L S A N D M E T H O D S

2.1 MATERIALS

2.1.1 General

All chemicals obtained for the preparation of solutions, including buffers, were from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of analytical grade or the highest grade obtainable at the time. In addition all solvents were from BDH Chemicals Ltd. and were used as obtained, with the exception of toluene which was redistilled before use.

2.1.2 Cell Culture

Mouse lymphoma cells, strain L1210, were obtained from Flow Laboratories, Irvine, Scotland.

RPMI 1640 medium, minimal essential Eagle's medium, L-glutamine (200mM), penicillin/streptomycin (5,000 I.U./ml and 5,000mcg/ml), foetal bovine serum (mycoplasma and virus screened), donor horse serum (mycoplasma and virus screened) and 7.5% (w/v) sodium bicarbonate solution for cell culture were from Flow Laboratories, Irvine, Scotland. Trypan blue (0.5% (w/v) in 0.85% (w/v) saline) was also from Flow Laboratories.

2.1.3 Radioactive Labels

[2-³H]adenosine, 24Ci/mmol and 21Ci/mmol, 1mCi/ml,
[methyl-³H]thymidine, 47.5Ci/mmol, 1mCi/ml,
[5-³H]uridine, 25.5Ci/mmol, 1mCi/ml,
L[4,5-³H]leucine, 53Ci/mmol, 1mCi/ml,
and [carbonyl-¹⁴C]nicotinamide, 59mCi/mmol, (made up to 100μCi/ml with 2% (v/v) EtOH) were all obtained from the

Radiochemical Centre, Amersham, Bucks., U.K.

[adenosine- ^3H]NAD $^+$ was prepared from [^3H]ATP (obtained from the Radiochemical Centre) by Dr. W.J.D.Whish, according to the method of Ohtsu & Nishizuka (1971) and had a final specific radioactivity of 20mCi/ μmol , 1mCi/ml.

2.1.4 Counting of Radioactive Samples

Toluene was from BDH Chemicals Ltd., Poole, Dorset, U.K., Triton X-100 from Sigma (London) Chemical Co., Poole, Dorset, U.K., 2,5-diphenyloxazole (PPO) from Packard Instrument Co., U.K. and glass fibre discs (GF/C) or paper discs (3MM qualitative, 2.4cm) from Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K.

2.1.5 Chromatography

(a) Gel filtration chromatography

Biogel P10 (50-100 mesh) was from BioRad Laboratories, Herts., U.K. and Sephadex G-75 (fine) and Blue Dextran 2000 were from Pharmacia Fine Chemicals, London, U.K.

Bovine serum albumin, cytochrome c and RNase A, used as molecular weight markers, were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and phenol red was from BDH Chemicals Ltd., Poole, Dorset, U.K.

(b) Ion-exchange column chromatography

Aminoethyl cellulose was from Sigma (London) Chemical Co., Poole, Dorset, U.K.

(c) Ion-exchange thin-layer chromatography

Polyethyleneimine cellulose (PEI-cellulose) thin-layer

sheets (20 x 20cm) were from J. T. Baker Chemical Co., Phillipsburg, New Jersey, U.S.A.

2.1.6 Caesium Chloride Density Gradient Centrifugation

Caesium chloride (grade I or optical grade) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., as was phenylmethysulphonyl fluoride (PMSF). Centrifugation of the samples was carried out in a Beckman L5-65 or L5-50 ultracentrifuge using 5ml cellulose nitrate tubes in an SW50.1 rotor.

2.1.7 Protein Estimation

Coomassie brilliant blue G-250 (a protein-binding dye) and bovine serum albumin (as standard) were both from Sigma (London) Chemical Co., Poole, Dorset, U.K.

2.1.8 DNA Estimation

Diaminobenzoic acid·2HCl (DABA) was obtained from Aldrich Chemical Co., U.K. and DNA (as standard, calf thymus type V) from Sigma (London) Chemical Co., Poole, Dorset, U.K.

2.1.9 NAD⁺ Assay

Thiazolyl blue (MTT) and phenazine methosulphate (PMS) were from Sigma (London) Chemical Co., Poole, Dorset, U.K. NAD⁺ and alcohol dehydrogenase (ADH) were from Boehringer Corporation, Lewes, Sussex, U.K. Nicotinamide was from BDH Chemicals Ltd., Poole, Dorset, U.K.

2.1.10 Substrates and Reagents

Standard markers for thin-layer chromatography of

5'AMP, 3'(2')AMP, adenosine and NAD^+ were from Boehringer Corporation, Lewes, Sussex, U.K., ADPR and 5'GMP from Sigma (London) Chemical Co., Poole, Dorset, U.K. and 5'dAMP from P-L Biochemicals, Berks., U.K.

Dithiothreitol (DTT), sodium dodecylsulphate (SDS), and 2'deoxycytidine were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Glucose-1-phosphate was from Boehringer Corporation, Lewes, Sussex, U.K.

2.1.11 Enzymes

Snake venom phosphodiesterase (from Crotalus adamanteus), EC 3.1.4.1, spleen phosphodiesterase (from bovine spleen), EC 3.1.4.18, alkaline phosphatase (from calf intestinal mucosa), EC 3.1.3.1, RNase A (from bovine pancreas), EC 3.1.4.22, DNase I (from bovine pancreas), EC 3.1.4.5, 5'-nucleotidase (from Crotalus adamanteus venom) EC 3.1.3.5 and 3'nucleotidase (from Rye grass), EC 3.1.3.6, were all obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Pronase (B.grade, from Streptomyces griseus), EC 3.4.21.4, was from Calbiochem, Herts., U.K.

2.1.12 Inhibitors

Thymidine was from Sigma (London) Chemical Co., Poole, Dorset, U.K. 3-aminobenzamide and 3-methoxybenzamide were synthesized as described in Purnell & Whish (1980b) by M. R. Purnell.

2.1.13 Cytotoxic Reagents

Dimethyl sulphate (DMS) was from Aldrich Chemical Co. Ltd., U.K.

2.1.14 Gel Electrophoresis

Acrylamide was from Fisons, Kent, U.K. and N,N¹-methylenebisacrylamide from BDH Chemicals Ltd., Poole, Dorset, U.K. Coomassie brilliant blue R-250 was from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Soluene 350 and Hyamine hydroxide, used in the scintillation cocktail, were from Packard Instrument Co., U.K. N,N,N¹,N¹-tetramethylethylenediamine (TEMED), ammonium persulphate and β -mercaptoethanol were from BDH Chemicals Ltd., Poole, Dorset, U.K. Molecular weight markers of bovine serum albumin, trypsinogen, ovalbumin and RNase A were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

2.2 METHODS

2.2.1 Cell Culture

(a) Aseptic procedure

To minimise contamination during cell culture it is necessary to employ aseptic technique. Hence, all glassware was sterilized before use by autoclaving at 15 lbs/in² for 20 minutes, as were any non-sterile solutions to be added to the growth medium or growing cells.

(b) The growth medium

Cells were grown in suspension culture in either RPMI 1640 medium or minimal essential Eagle's medium supplemented with glutamine, penicillin/streptomycin, sodium bicarbonate and foetal bovine serum or donor horse serum.

The composition of the medium/100mls is shown in Table 2.2.1

Table 2.2.1

Quantity (ml)	Component
10	10x medium
74.3	double-distilled H ₂ O
1	L-glutamine
2	penicillin/streptomycin
2.7	sodium bicarbonate
10	serum

Once the medium was complete it was stored at 4°C and discarded if not used within a week.

(c) Subculturing and counting of cells

During the growth of cells in suspension culture a number of factors such as change in pH, production of toxic wastes and exhaustion of nutrients may render the medium unsuitable for further cell propagation. Thus, to ensure that cells remain viable and healthy it is necessary to transfer them to fresh medium once, or before, they have grown to high density.

The density and hence growth of cells may be determined by counting the cells under the microscope, using a haemocytometer. In this way the number of cells/ml may be calculated and with a knowledge of the doubling time (see Chapter 3) it was possible to subculture cells to the required density. This was carried out by placing an aliquot of cells suspended in old medium into a sterile

bottle and adding warm, fresh medium of defined volume. Cells were routinely subcultured to a density of 1×10^5 cells/ml. They were then gassed with 95%/5% (v/v) air/CO₂ mixture through a Millipore filter, and incubated at 37°C.

Microscopic examination of the cells during counting also enabled the detection of contamination or the presence of old and non-healthy cells.

(d) Cell viability

Cell viability was determined using a solution of trypan blue. The dye is absorbed by and thus stains dead but not healthy cells. To 1ml cell suspension 0.5ml trypan blue was added, the two mixed thoroughly, and an aliquot added to a haemocytometer for examination under the microscope. Viability was given by the equation

$$\frac{\text{Total number of cells} - \text{number of dead cells}}{\text{Total number of cells}} \times 100\%$$

2.2.2 Preparation of Samples for Counting
by Trichloroacetic Acid Precipitation

Acid-insoluble material was prepared for counting in either one of two ways: (a) on glass-fibre discs (b) on paper discs.

(a) TCA was added to a given volume of sample to give a final concentration of 20% (w/v) and the mixture left on ice for 30 minutes. The precipitated sample was then added to a GF/C disc (using an ultrafiltration tower) prewashed in 5ml 20% (w/v) TCA. The disc was washed three times with 5ml 5% (w/v) TCA, once with 5ml 95% (v/v)

ethanol, and dried for 20 minutes in an oven at 70°C before counting.

(b) To Whatman 3MM paper discs prewashed in 20% (w/v) TCA in ether for 30 minutes, 5-20µl sample were added and the discs allowed to dry. Discs were then washed in 20% (w/v) TCA for 30 minutes, keeping on ice, then in each of the following for 5 minutes - 20% (w/v) TCA, 95% (v/v) ethanol (2x), and diethyl ether, and allowed to dry before counting. Approximately 2ml of solution or solvent was used for each disc.

The advantage of the first method is that a greater volume of sample may be counted and that of the second that a large number of samples may be processed rapidly.

2.2.3 Counting of Radioactive Samples

Two isotopes were used in these studies - ^3H and ^{14}C . Radioactivity was determined by liquid scintillation counting, using a Packard (Tri-Carb) liquid scintillation spectrometer.

Essentially, samples were divided into two categories:
(a) aqueous material, (b) insoluble material.

(a) Aqueous samples

Aqueous samples were counted in Triton/toluene/PPO scintillator (see Table 2.2.3), using 10 parts scintillant to 1 part sample. Counting efficiencies were determined by counting 5µl of the radioactive label used. Average efficiencies of 28% for ^3H and 67% for ^{14}C were obtained.

(b) Insoluble samples

Insoluble samples were collected on glass fibre (GF/C) or paper discs and counted in 2ml toluene/PPO scintillator (see Table 2.2.3). When using paper discs it was ensured that the discs were placed with the pipetted sample spot face upwards. Counting efficiencies were determined by counting 5 μ l of [3 H]poly(ADPR) on a disc. Using 3 H, average efficiencies of 37% and 38% were obtained on GF/C and 3MM, respectively. Using 14 C, an efficiency of 70% was obtained on GF/C.

Table 2.2.3

	Triton/toluene scintillant	Toluene scintillant
Toluene	700ml	1,000ml
Triton X-100	300ml	-
2,5-diphenyloxazole	5g	5g

2.2.4 Isolation of Pig Thymus Nuclei

The method was a modification of that of Khan & Shall (1976). To 25g frozen pig thymus on ice, 210ml of an ice-cold solution of 0.32M sucrose, 3mM MgCl₂, 12mM triethanolamine/HCl-pH 7.5 were added. The mixture was homogenized in a Sorval homogenizer for 3 x 30 seconds at position 2.5, filtered through one thickness of muslin and centrifuged at 2,500g for 15 minutes at 4°C to pellet the nuclei. Nuclei were then resuspended in 5ml of a solution containing 0.5M NaCl, 4mM KF, 60mM KCl, 10mM MgCl₂, 100mM

triethanolamine/HCl-pH 8.2, 17mM DTT. The mixture was then centrifuged for 1h in a Beckman ultracentrifuge at 150,000g, using an SW50.1 rotor at 4°C. The pellet, containing nuclei, was used for subsequent studies.

2.2.5 Isolation of Mouse L1210 Nuclei

1×10^7 cells were centrifuged at 300g for 10 minutes at 4°C. The pellet was then resuspended in 20ml hypotonic cell wash solution containing 4mM MgCl₂, 1mM Tris/HCl-pH 7.9, 0.3mM EDTA, 0.02% (v/v) β -mercaptoethanol and 1mM CaCl₂, and the mixture left on ice for 10 minutes. The sample was then recentrifuged at 300g for 10 minutes at 4°C and resuspended in 20ml of a solution containing 0.32M sucrose, 0.003% (v/v) Triton N101, 1mM MgCl₂, 1mM Tris/HCl-pH 6.4, 0.02% (v/v) β -mercaptoethanol and 1mM CaCl₂. The mixture was homogenized in a Teflon homogenizer (6 strokes) and recentrifuged at 300g for 10 minutes at 4°C. The crude nuclei were then washed twice in 20ml of a solution containing 4mM KF, 60mM KCl, 10mM MgCl₂, 100mM triethanolamine/HCl-pH 8.2, 0.5mM DTT, centrifuging as above.

2.2.6 [³H]NAD⁺ Incorporation into Isolated Nuclei

The method was essentially that of Nishizuka et al. (1967). 20 μ l (5×10^6) sample nuclei were resuspended in 0.46ml of a solution containing 4mM KF, 60mM KCl, 10mM MgCl₂, 100mM triethanolamine/HCl-pH 8.2, 0.5mM DTT, 20 μ l [³H]NAD⁺ (1 nmol) added and the mixture incubated at 26°C for 10 minutes. The reaction was stopped by either (a) centrifuging twice

in 0.15M NaCl at 800g at 4°C, or (b) by addition of TCA to 20% (w/v) and left on ice for 30 minutes.

2.2.7 Biogel P10 Chromatography of [³H]NAD⁺-Labelled Pig Thymus Nuclei

Biogel P10 column chromatography of [³H]NAD⁺-labelled nuclei was carried out to remove excess label which might interfere with further analyses. A column of dimensions 25cm x 3.14cm² was allowed to equilibrate in a solution containing 0.1M NaAc/HAc-pH 6.5, 4M urea, 0.5mM NaN₃ at a flow rate of 20ml/h.

Labelled nuclei were then applied to the column in the presence of Blue Dextran 2000 and the sample eluted. 1ml fractions were collected and 200μl samples counted for total and acid-insoluble radioactivity.

Calibration of the column gave a good indication of the expected elution volume, V_e , of the sample molecules. Blue Dextran 2000 was used to determine the void volume, V_o , of the column and bovine serum albumin (mol.wt.66,000), cytochrome c (mol.wt.11,700) and phenol red (mol.wt.362) used to determine the relative positioning of NAD⁺ and of ADP-ribosylated protein and to determine the approximate molecular weight range of the ADP-ribosylated protein.

2.2.8. [³H]Adenosine Incorporation by Mouse L1210 Cells

5ml cells in a McCartney bottle, at a density of 2×10^5 /ml were labelled with [³H]adenosine at a concentration of 40μCi/ml. Labelling was carried out for 18h at 37°C.

2.2.9 Harvesting of Cells Radioactively Labelled with [³H]Adenosine

The procedure finally adopted and described below was found to give maximum recovery of acid-insoluble material and removal of excess label (see Chapter 3).

Cells were taken, medium aspirated off and the cells quickly frozen in liquid nitrogen. Before thawing TCA was added to a final concentration of 20% (w/v) and the mixture left on ice for 1h. The precipitate was centrifuged at 800g for 20 minutes at 4°C. The pellet was then washed once in 20% (w/v) TCA and twice in 70% (v/v) acetone, 50mM NaAc/HAc-pH5, centrifuging as above.

2.2.10 Caesium Chloride Density Gradient Centrifugation of [³H]-Labelled Cells

The pellet obtained at the final stage of harvesting was resuspended in 2.5ml 10M urea and left for 15 minutes at 15°C to allow for complete denaturation of isolated material. 2.5ml of a solution containing 100% (w/v) CsCl, 0.2M NaAc/HAc-pH5, 0.2mM PMSF were then added, the solution thoroughly mixed and left for a further 15 minutes.

Density gradient centrifugation was carried out at 200,000g (r_{av}) for 72h at 15°C in a Beckman L5-65 or L5-50 ultracentrifuge using an SW50.1 rotor. After centrifugation samples were fractionated from the bottom upwards using a syringe needle carefully lowered to the bottom of the gradient (Fox & Pardee, 1970) and 6 drop fractions (about 0.25ml) collected at the rate of 12ml/h. Samples

were counted for radioactivity after TCA precipitation, as previously described. The density profile of the gradient was determined by measuring the weight of an 0.1ml sample of each fraction and correcting for urea. This method was routinely used for the isolation of ADP-ribosylated protein from DNA and RNA on the basis of buoyant density (see Chapter 3).

2.2.11 Processing of Isolated [³H]Adenosine-Labelled Material

Relevant fractions from the top of the gradient were freed of salt by precipitation in 20% (w/v) TCA overnight. The precipitate was centrifuged at 3,000g for 20 minutes in a bench centrifuge, then washed once in 20% (w/v) TCA and twice in 70% (v/v) acetone, 50mM NaAc/HAc-pH5, centrifuging as above.

2.2.12 Enzymic and Chemical Analyses of Isolated [³H]Adenosine-Labelled Material

[³H]adenosine-labelled material isolated by caesium chloride density gradient centrifugation was routinely analysed by the following:

(a) Digestion by snake venom phosphodiesterase

To 25µl sample in 5M urea 175µl of a solution containing 4M urea, 50mM Tris/HCl-pH 8.2, 20mM glucose-1-phosphate, 20mM MgCl₂, and 0.2 I.U. of the enzyme were added and the sample incubated at 37°C for 6h. The enzyme is stimulated by MgCl₂ (Razzell & Khorana, 1959). Glucose-1-phosphate was added to inhibit contaminating traces of nucleotide pyrophosphatase. Glucose-1-phosphate was

chosen rather than 5'AMP or 5'GMP because the latter are end products of the phosphodiesterase digest and may thus cause end-product inhibition.

(b) Alkaline phosphatase digestion of phosphodiesterase digest

To 50 μ l of the snake venom phosphodiesterase digest 150 μ l of a solution containing 50mM Tris/HCl-pH 7.5, 10mM MgCl₂, and 0.2 I.U. alkaline phosphatase were added and the mixture incubated at 37°C for 2h. MgCl₂ stimulates the phosphatase (Schüssler, 1968).

(c) Base hydrolysis

To 25 μ l of sample in 5M urea 175 μ l 0.1M NaOH were added and the mixture incubated at 37°C overnight.

2.2.13 Ion-exchange Thin-Layer Chromatography

(a) Preparation of plates

All thin-layer chromatography was carried out using polyethyleneimine cellulose (an anion-exchanger) thin-layer sheets. Before use sheets were soaked in 2M NaCl for 30 minutes, rinsed thoroughly in distilled water and then left in distilled water for a further hour, before allowing to dry at room temperature. In this way, any contaminating material (e.g. salt) was removed.

(b) Application of standard markers and sample

Standards for t.l.c. were made up in 25% (v/v) or 50% (v/v) ethanol at a concentration of 5mg/ml solution.

10 μ l standard and 10-50 μ l sample per cm were plated on the origin which was positioned 2cm from the bottom of the plate. The origin was dried in a stream of warm air after each addition of standard or sample.

If the applied sample contained a high concentration of salt then the plate was first soaked in anhydrous methanol for at least 15 minutes and dried before development (Stone et al., 1973). After development in the desired system the plate was dried in a stream of warm air. Analysis of the separation was made by observing the position of the standard markers under a uv light source of emission 250-280nm.

(c) T.l.c. systems

The following t.l.c. systems were used routinely:

1. This system was used when a good separation of ADPR/PR-AMP/AMP was required. Separation is mainly achieved according to the phosphate and base moieties of the compound. After application of the sample the plate was placed in a chromatographic tank containing 50ml 1.0M HAC and the solvent front allowed to run 2cm above the origin. The t.l.c. plate was then transferred, without intermediate drying, to a tank containing 50ml 0.9M HAC/0.3M LiCl and the solvent front allowed to run a further 13cm. The separation obtained can be seen in Fig. 2.2.13.1. R_f values for ADPR, 5'GMP, 5'AMP and adenosine were 0.3, 0.4, 0.57 and 0.87, respectively. PR-AMP runs just a little ahead, but overlapping, 5'GMP. The system was routinely used for analysis of snake venom phosphodiesterase digests, where

Fig. 2.2.13.1 1M HAC/0.9M HAC - 0.3M LiCl T.l.c. of Snake Venom Phosphodiesterase Digest

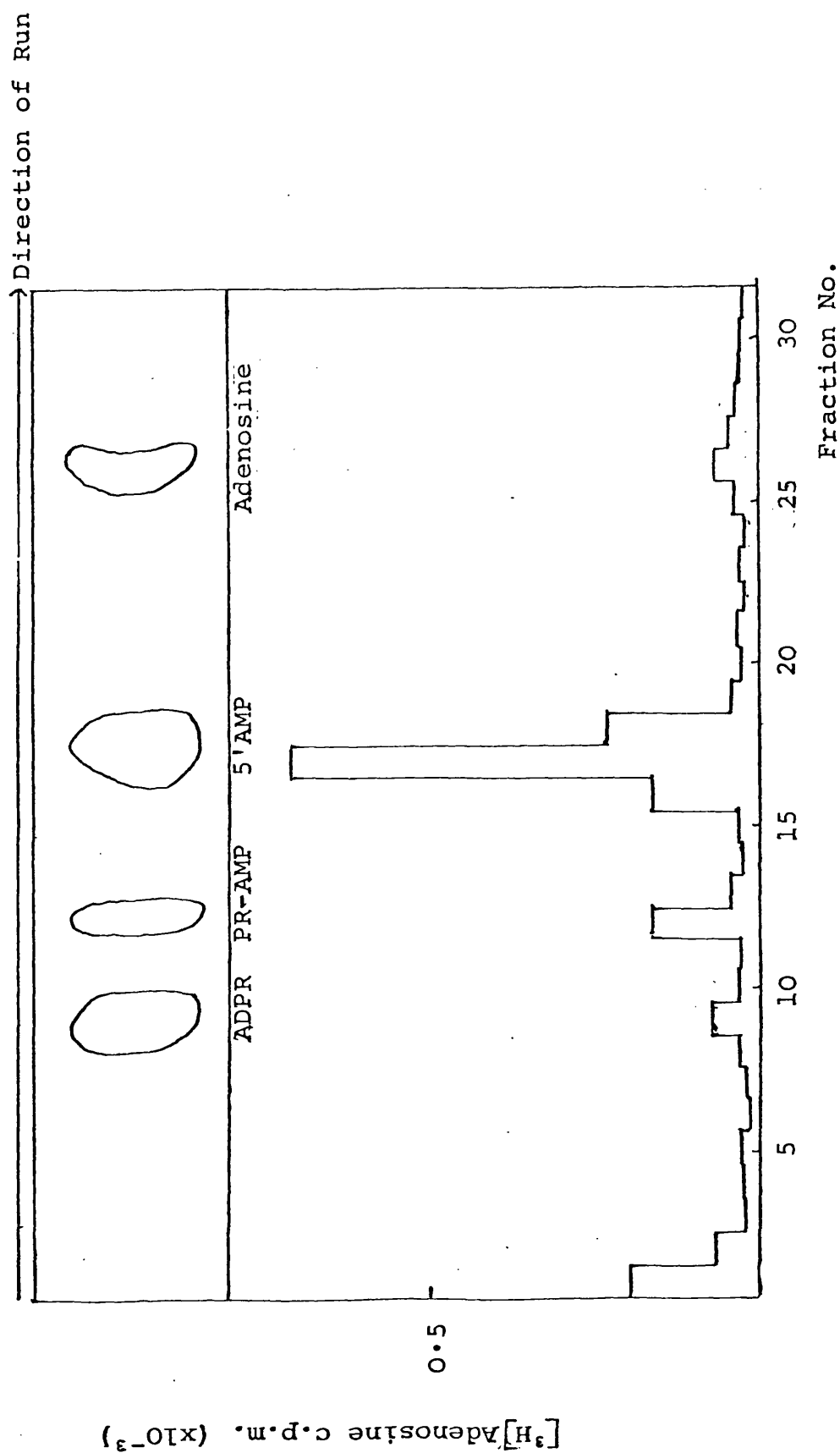
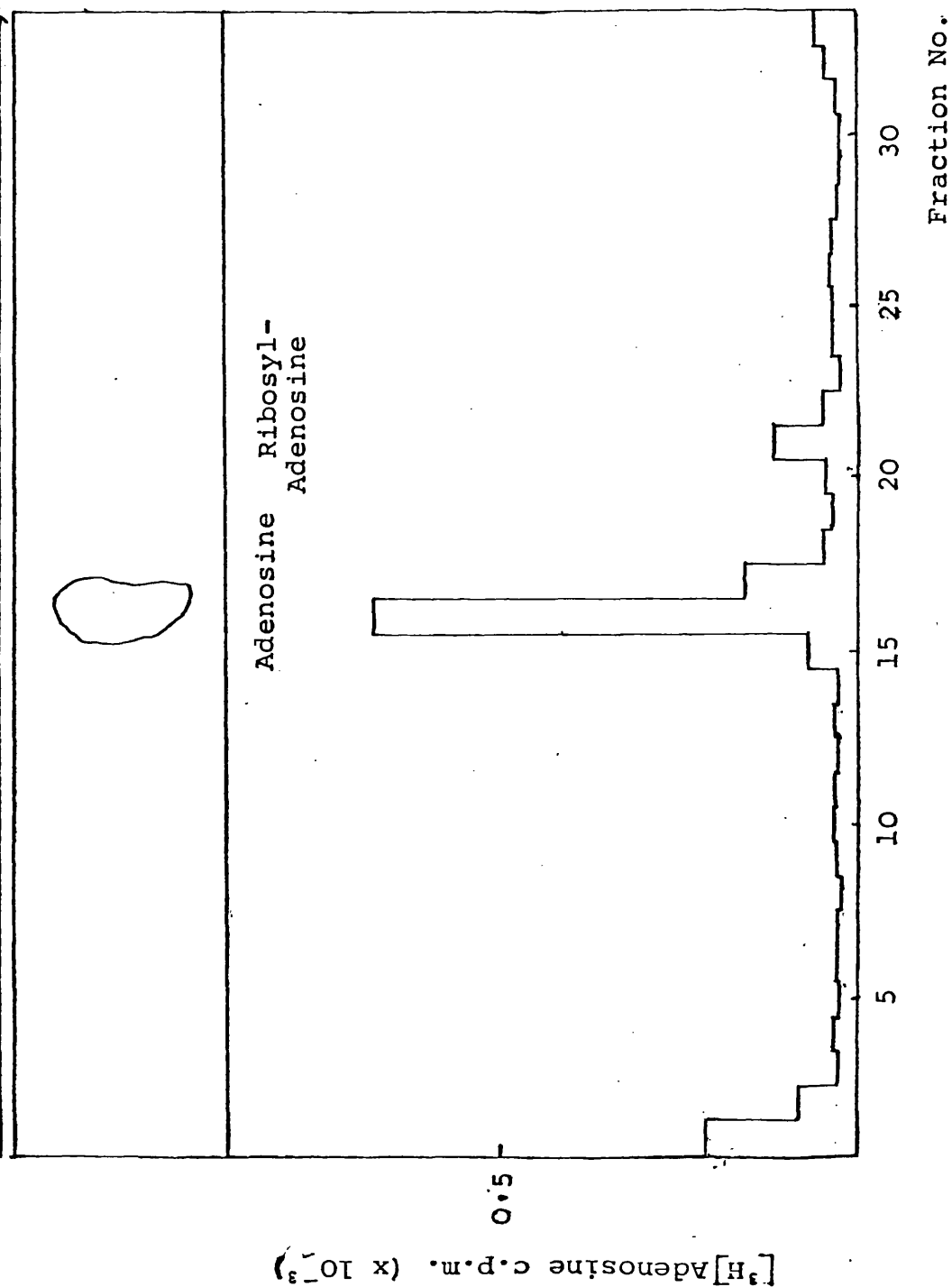


Fig. 2.2.13.2 Double-Distilled Water T.l.c. of Alkaline Digest

Direction of Run



PR-AMP and 5'AMP are produced (see Chapter 4). The method is that of Randerath & Randerath (1965a).

2. This system was used for separation of ribosyl-adenosine and adenosine, products of further digestion of the snake venom phosphodiesterase digest by alkaline phosphatase (see Chapter 4). The system separates nucleosides according to the sugar moieties of the molecule (Miwa et al., 1979). Any nucleotides remain at or near the origin. After application of the sample the t.l.c. plate was placed in a chromatographic tank containing 50ml double-distilled water and the solvent allowed to run 16 cm above the origin. The separation achieved may be seen in Fig. 2.2.13.2. R_f values for ribosyl adenosine and adenosine were 0.66 and 0.5, respectively.

3. This system was used to achieve separation of 5'AMP and 3'AMP, products of base digestion of mono(ADPR)-protein and RNA, respectively (see Chapter 4). The separation of 5'AMP and 3'AMP is achieved by the fact that 5'AMP, but not 3'AMP, is capable of forming a cis-diol complex with borate, due to free hydroxyl groups at the 2' and 3' positions of the sugar moiety of the molecule (see Fig. 2.2.13.3). The formation of this complex retards the mobility of the molecule during t.l.c. After application of the sample the plate was placed in a chromatographic tank containing 50ml 2M LiCl/absolute EtOH in the ratio 1:1 (by vol.), saturated with H_3BO_3 and adjusted to pH7 with ammonia solution. The solvent front was allowed to run at least 9.5cm above the origin.

Fig. 2.2.13.3 Borate Complex Formation with Sugars
Containing Free Hydroxyl Groups at
the 2' and 3' Positions

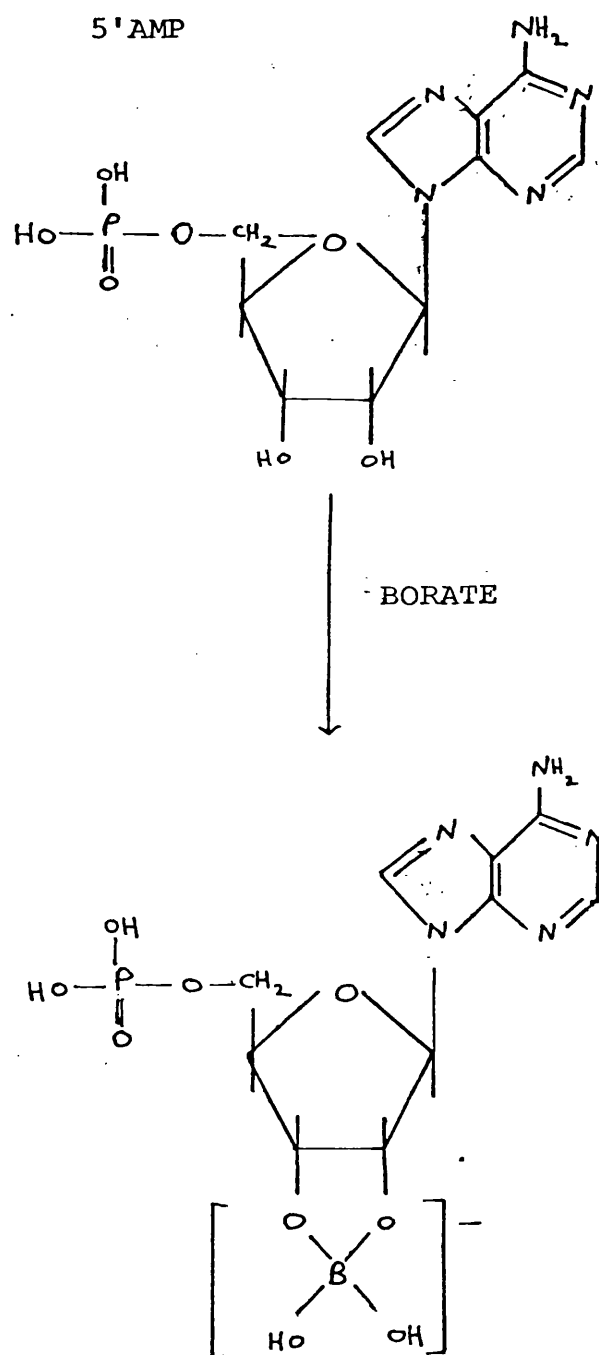


Fig. 2.2.13.4 LiCl/EtOH/(NH₄)₂BO₃ T.l.c.
of Base Digest

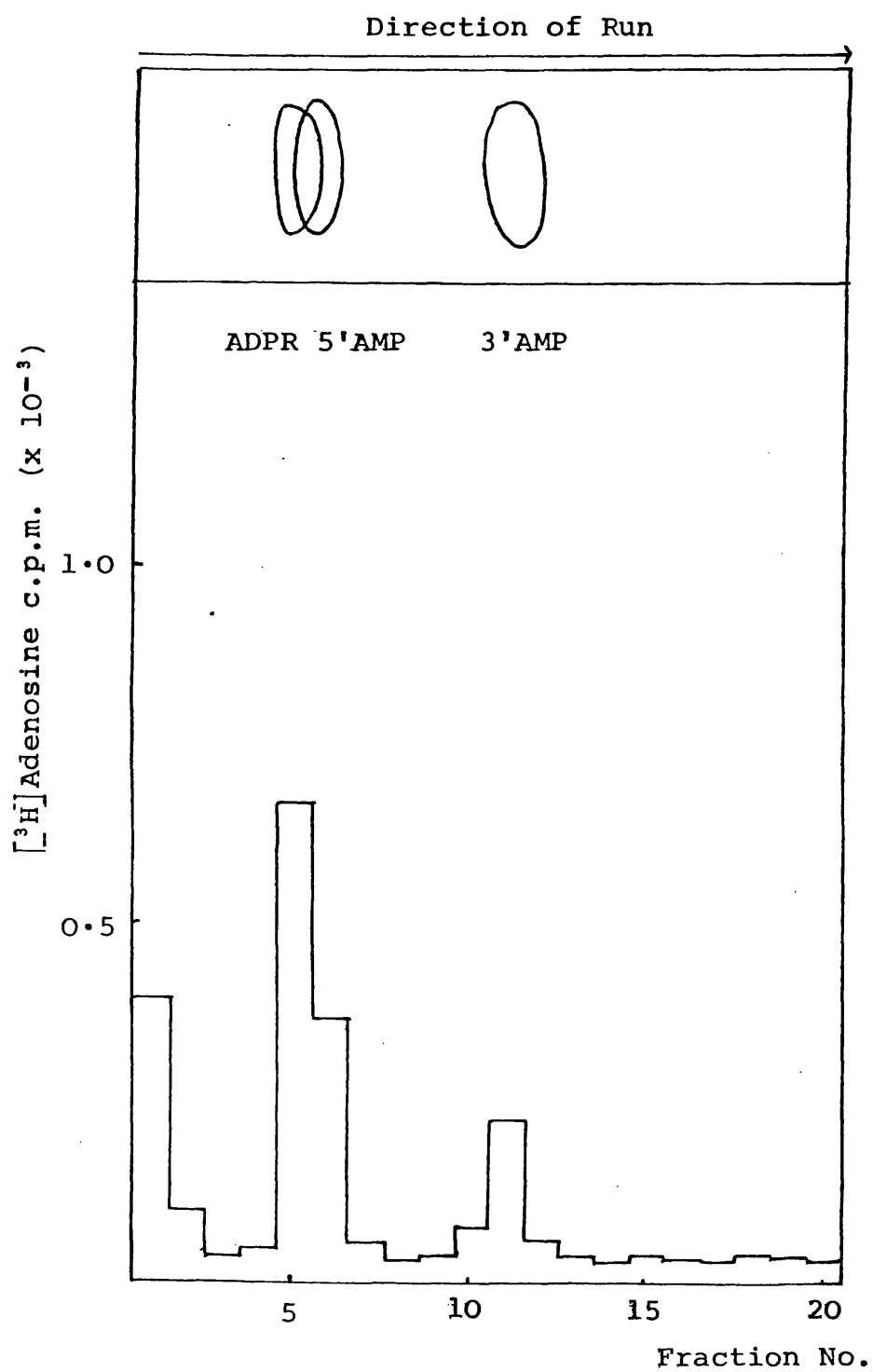
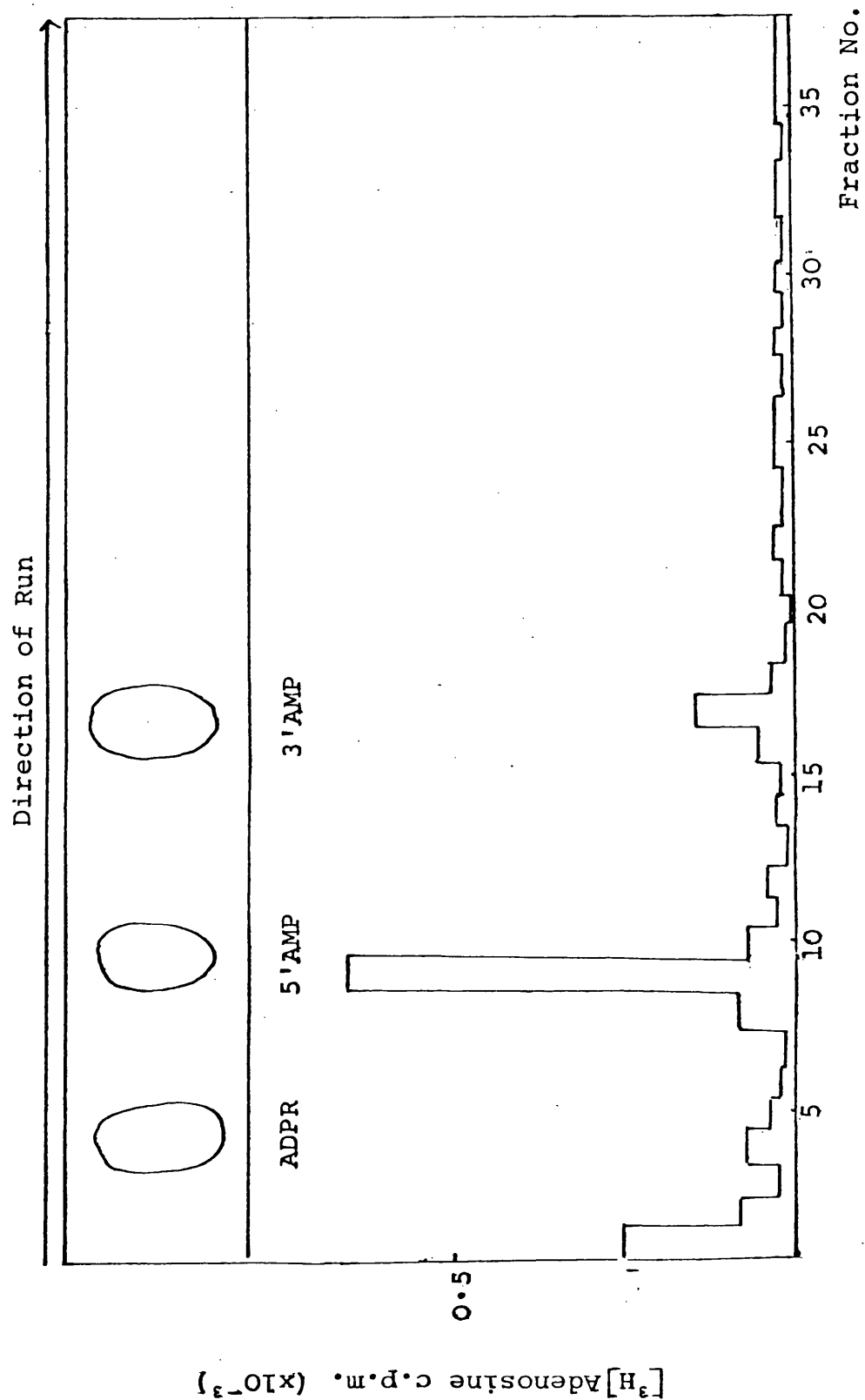


Fig. 2.2.13.5 0.15M Sodium Tetraborate/0.5M Boric Acid T.l.c. of Base Digest



The separation achieved may be seen in Fig.2.2.13.4. R_f values for ADPR, 5'AMP, 3'AMP and 5'dAMP were 0.29, 0.29, 0.58 and 0.58, respectively (Schwartz & Drach, 1975).

4. This system was used to achieve separation of not only 5'AMP and 3'AMP, as above, but also ADPR, an intermediate product of base digestion of protein-bound ADPR (Goebel et al., 1977). Separation is achieved on much the same basis as System 3. After application of the sample the plate was placed in a tank containing 50ml 0.15M sodium tetraborate/0.5M boric acid and the solvent front allowed to run to the top of the plate i.e. 18cm. The separation obtained may be seen in Fig. 2.2.13.5. R_f values for ADPR, 5'AMP and 3'AMP were 0.11, 0.25 and 0.46, respectively (Young & Sweeney, 1978). This system has two advantages over system 3: (i) better separation of products of base digestion is achieved, (ii) the time to run the system is shorter.

(d) Counting of t.l.c.s.

The plate was cut into 0.5cm strips, each strip scraped into a vial and 200 μ l 1.6M LiCl added to elute the sample. 2ml of Triton/toluene/PPO scintillant were added and the samples counted for radioactivity. Efficiency of counting was 25%. Blank values averaged 30 c.p.m.

2.2.14 Protein estimation

Protein estimation was made using the protein dye-binding assay developed by Bradford (1976). This utilizes the binding of Coomassie brilliant blue G-250 to protein. Under slightly acidic conditions the dye anion is electro-

statically attracted towards the NH_3^+ groups of the protein. This binding causes a shift in the absorption maximum of the dye from 465 to 595nm and a consequent colour change from red to blue of the solution. It is this increase in absorption at 595nm which is monitored. Because the dye-protein complex is formed within two minutes and stable for one hour, the assay allowed the processing of a large number of samples at any one time. In addition, the readings were not affected by any of the reagents, e.g. 5M urea, present in the protein sample. A standard curve was set up each time, using bovine serum albumin in the range 10-100 μg .

The protein reagent was prepared by dissolving 100mg Coomassie brilliant blue G-250 in 50ml 95% (v/v) EtOH. 100ml 85% (w/v) phosphoric acid were then added and the mixture diluted to a final volume of 1 litre. The final concentrations of the reagents in the solution were 0.01% (w/v) Coomassie brilliant blue G-250, 4.75% (v/v) EtOH and 8.5% (w/v) phosphoric acid.

50 μl of protein sample were made up to 0.1ml with 0.1M Tris/HCl-pH 7.5 and 5ml of the protein reagent added. The solution was thoroughly mixed and after a minimum of 5 minutes the absorbance at 595nm was measured in a Cecil SP202 spectrophotometer.

2.2.15 DNA Estimation

DNA estimation was made using the colorimetric assay of Setaro & Morley (1977). This method utilizes the reaction of DABA \cdot 2HCl with DNA by measuring the optical

density of the DABA-deoxyribose product at 420nm. Deoxyribose is liberated from DNA by acid hydrolysis using perchloric acid (PCA). The aldehyde ($R-CH_2-CHO$) nature of the liberated deoxyribose allows it to react with DABA under prolonged heating at high temperatures in concentrated mineral acid to yield a strongly fluorescent product. The reaction is specific for deoxypentoses and requires the carbon atom in the α position to the aldehyde group to be unsubstituted. A standard curve was set up using calf thymus DNA in the range 25-1,000 μ g.

To 50 μ l sample 50 μ l 2M PCA were added and the sample left at room temperature for 10 minutes. 0.1ml 1.32M DABA was then added, the mixture incubated at 60°C for 30 minutes and then made up to a final volume of 2ml with 0.6M PCA. Optical density of the solution was then measured at 420nm in a Cecil SP202 spectrophotometer.

2.2.16 NAD⁺ Assay

NAD⁺ was assayed according to the method of Nisselbaum & Green (1969). When NAD⁺ is added to a suitably buffered mixture of phenazine methosulphate (PMS), thiazolyl blue (MTT), ADH and ethanol, the following reaction occurs,



The NADH produced reduces MTT, through the intermediation of PMS, to the corresponding purple-coloured formazan. The rate of reduction of MTT is proportional to the concentration of NAD⁺.

A standard curve in the range 0.05-1.0 μ g NAD⁺ was set

up. The reaction mixture contained 0.008% (w/v) MTT, 0.027% (w/v) PMS, 0.003% (w/v) ADH, 42mM glycylglycine buffer-pH 7.4, 65mM nicotinamide and 325mM EtOH. The mixture was incubated for 10 minutes at 37°C and the reaction initiated by addition of standard NAD⁺ solution in the ratio of 1:29 (by vol.). Absorbance at 556nm was measured in a Pye Unicam SP8-100 uv spectrophotometer.

For assay, mouse L1210 cells were harvested by centrifuging at 300g for 10 minutes at 4°C. 0.5ml 50% (v/v) EtOH was added, the mixture sonicated (2 x 10 seconds) and then left on ice for 3h. The sample was then centrifuged at 10,000g for 10 minutes at 4°C and the supernatant analysed for NAD⁺ content as described for standards above.

2.2.17 Aminoethyl Cellulose Chromatography

This was routinely used for the separation of monomeric and polymeric material obtained from base digestion (see Chapter 4). Aminoethyl cellulose (an anion-exchanger) was swollen in 6M HAc and packed into a 2ml syringe to form a column of dimensions 2cm x 0.785cm². The column was equilibrated and the lyophilized base digest, in 100μl distilled water, applied. AMP and ADPR were eluted with 10ml 6M HAc. The column was then washed with 5ml distilled water and poly(ADPR) eluted with 10ml 1M ethylamine. 1ml fractions were collected and 500μl samples counted in Triton/toluene/PPO scintillant. An equal volume of 10% (v/v) HCl was added to the ethylamine fraction prior to the addition of scintillant to counteract

for any chemiluminescence. Recovery from the column was quantitative.

2.2.18 SDS Gel Electrophoresis

Electrophoresis involves the application of an electric field to the separation of proteins. Separation usually occurs in a stabilizing medium and here polyacrylamide gel was used. Acrylamide was polymerized using N,N'-methylenebisacrylamide as cross-linking agent, ammonium persulphate as initiator and TEMED as catalyst.

(a) Electrophoresis

SDS gel electrophoresis was employed to analyse proteins from the top of the gradient. SDS is an anionic detergent which disrupts hydrophobic interactions of proteins to produce rod-like complexes of identical charge density (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanfold, 1970,a,b). Subsequent electrophoresis thus allows separation solely on the basis of molecular weight. The method employed was a modification of that of Weber & Osborn (1969). The pH was lowered to 6 because of the lability of the ADPR-protein bond at a pH greater than 7 (Nishizuka et al., 1969). A discontinuous phosphate buffer system allowed a high degree of resolution. Urea was present in the gel in addition to SDS and, as a result, decreased the pore size of the gel.

Gels contained 7.8% (w/v) acrylamide, 0.18% (w/v) methylenebisacrylamide as crosslinker, 0.1% (w/v) SDS, 5M urea and 0.2M sodium phosphate buffer - pH6 (0.2M

with respect to phosphate). (In addition, it contained 10^{-4} mM ammonium persulphate and 0.0015% (v/v) TEMED). The gels were formed in glass tubes of length 10cm and internal diameter 4mm. The gel solution was degassed before pouring and the top of the gel protected from oxygen by a layer of water which also served to level the surface.

Samples were dissolved in 10M urea at a concentration of 2.6mg/ml and incubated at room temperature for 30 minutes. The solution was then diluted in buffer to 1mg/ml such that the final solution contained 3.8M urea, 7.7mM sodium phosphate buffer-pH6 (7.7mM with respect to phosphate), 0.08% (w/v) SDS, 0.08% (v/v) β -mercaptoethanol, 8% (w/v) glycerol and 0.008% (w/v) bromophenol blue. Incubation was continued for a further 2.5h at room temperature. Pretreatment with urea alone was necessary to achieve solubilization of protein. Reduction of disulphide bridges with β -mercaptoethanol was carried out at room temperature since the protein-ADPR linkage is heat-labile.

The electrode buffer was 0.1M sodium phosphate-pH6, containing 0.1% (w/v) SDS and 5M urea.

All solutions containing urea were freshly made up before use to minimise the generation of cyanate from urea, which may alter the conditions of electrophoresis.

Samples (20 μ l) were applied to the top of the gel under the electrode buffer, using a Hamilton syringe. Electrophoresis was carried out at 3mA/gel for the first 10 minutes and then at 8mA/gel. Gels were removed,

after electrophoresis, by using a syringe to rim around the gel with water to loosen contact with the surface of the tube.

(b) Counting of gels

Gels were counted according to the one-step method of Aloyo (1979). Gel slices (approx. 0.15cm) were placed into 10ml scintillant containing 0.6% (w/v) PPO, 1% (v/v) Soluene 350 and 1% (v/v) Hyamine hydroxide in toluene. Incubation was continued for 48h at room temperature with occasional shaking and gel slices counted in a Packard scintillation spectrometer. Counting efficiency was 30%.

(c) Staining of gels

Gels were stained overnight in 0.26% (w/v) Coomassie brilliant blue R-250, in methanol/glacial HAc/water (4:1:4, by vol.). The Coomassie brilliant blue binds to proteins in the same way as described in Section 2.2.14.

Destaining was carried out at 37°C in glacial HAc/methanol/water (7:5:88, by vol.).

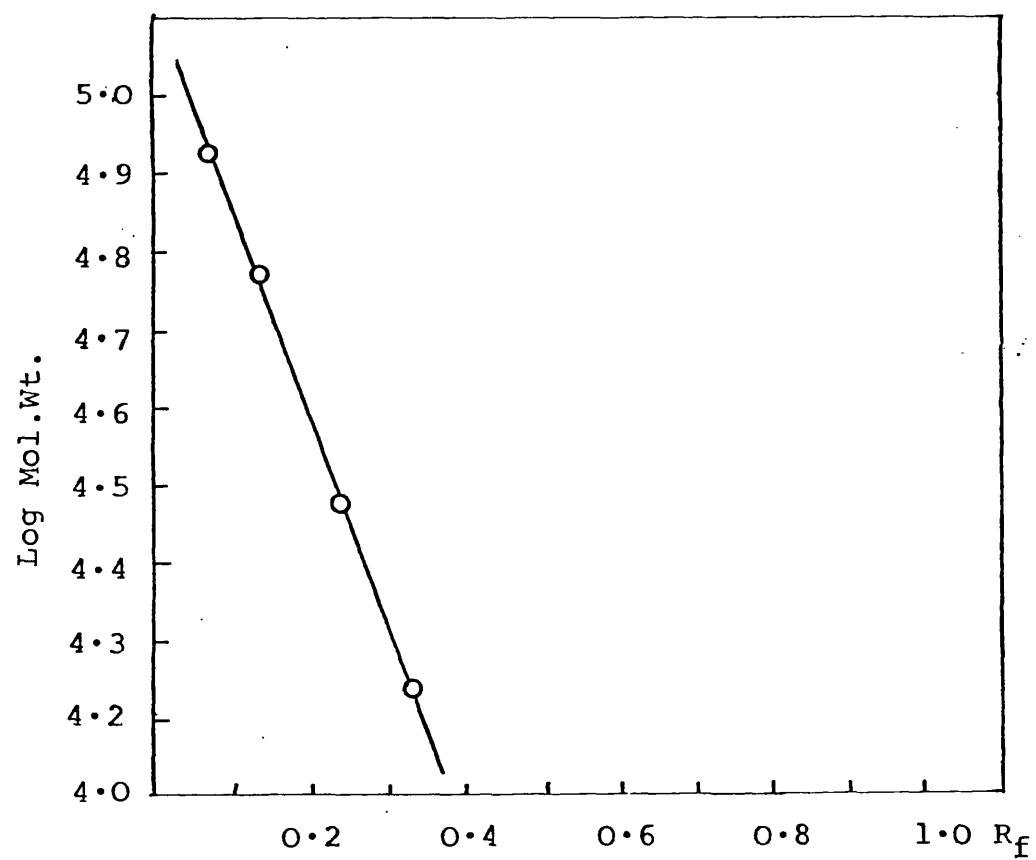
(d) Molecular weight estimation

Relative mobilities of protein bands were defined as

$$R_f = \frac{\text{distance migrated by protein band}}{\text{distance migrated by bromophenol blue marker}}$$

Gels were calibrated for molecular weight by simultaneous electrophoresis of a mixture of marker proteins containing bovine serum albumin (mol.wt. 66,000), ovalbumin (mol.wt. 45,000), trypsinogen (mol.wt. 24,000)

Fig. 2.2.18 Standard Curve for Molecular Weight
Estimation on SDS Polyacrylamide Gels



and RNase A (mol.wt. 13,700]. The mixture contained 1mg/ml of each protein.

The calibration curve was plotted as log mol.wt. of protein against the relative mobility. A good straight line relationship was obtained, in agreement with the findings of Shapiro et al. (1967) and Weber & Osborne (1969). (Fig. 2.2.18).

(e) Scanning of gels

Gels were scanned using the gel scanning attachment of a Pye-Unicam SP 1800 uv spectrophotometer. Gels were scanned at 600nm using a slit width of 1mm and the scans recorded with a Pye-Unicam Linear Recorder. Scanning allowed a more quantitative assessment of the pattern of protein distribution to be made compared with that of direct visualization.

CHAPTER 3

A METHOD FOR THE
ISOLATION OF PROTEIN
ADP-RIBOSYLATED IN VIVO

3.1 INTRODUCTION

The majority of studies on poly(ADPR) and the ADP-ribosylation of nuclear proteins have been carried out in vitro for two reasons: (i) the immediate precursor of the reaction, NAD^+ , cannot enter the cell (Nolde & Hilz, 1972), and (ii) levels of poly(ADPR) in the cell are low compared to those of DNA and RNA. This precludes a straightforward isolation and analysis of polymer using radioactive precursors less specific than NAD^+ . These problems are overcome by using isolated nuclei. However, such a system brings problems in itself; ADP-ribosylation in vitro appears to be heterogeneous not only in terms of the activity of the synthetase during various nuclear events, but also in the protein species modified and in the nature of the linkage of ADPR to protein. Much of this heterogeneity may arise from the actual experimental conditions employed and may account for some of the differences observed even when the role of ADP-ribosylation with respect to one particular nuclear function is studied. In fact, most of the conditions used for isolating nuclei are likely to alter the state of the chromatin and may even cause fragmentation of DNA (Halldorsson et al., 1978), an event in itself capable of causing alterations in chromatin structure and function.

In an attempt to overcome these problems Berger et al. (1978a) and Halldorsson et al. (1978) developed permeabilized cell systems in which NAD^+ can enter the cell and subsequently become incorporated into ADP-ribosylated protein. The system was further characterized and compared

with isolated nuclei. Halldorsson et al. (1978), using mouse L5178Y cells, showed by alkaline sucrose density gradient centrifugation that the DNA of isolated nuclei is fragmented whereas that of permeabilized cells is indistinguishable from that of untreated cells. Berger et al. (1977) using mouse L cells showed that DNA synthesis in permeabilized cells occurs at a much greater rate and for a longer period of time than that in isolated nuclei. In addition, the DNA synthesized by the permeabilized cells reaches much higher molecular weight than that synthesized by isolated nuclei, and is, in fact, within the size range of eukaryotic replicons (Huberman et al., 1968; Gautschi et al., 1973). DNA synthesized in isolated nuclei is comparable to Okazaki pieces but no greater. Berger et al. (1977) also demonstrated that DNA synthesis in permeabilized cells is a continuation of that initiated in vivo and that it is semiconservative (Berger & Johnson, 1976). The observed arrest of DNA synthesis after 20-30 minutes under these conditions is probably due to the absence of initiation of new replicons in permeabilized cells (Berger et al., 1977).

Using permeabilized cells, Halldorsson et al. (1978) showed that the activity of poly(ADPR) synthetase is low in these cells compared to that in isolated nuclei and that the high activity of the enzyme in the latter is an artifact of nuclear isolation procedures, probably a result of DNA fragmentation. In addition, Berger & Johnson (1976) showed that poly(ADPR) synthesis in permeabilized cells occurs for a longer period of time

than that in isolated nuclei; the permeabilized cell system appeared to more closely approximate the system in vivo than did isolated nuclei. However, Halldorsson et al. (1978) demonstrated that, during incubation at 26°C in isotonic buffer, the DNA of permeabilized cells is degraded. Furthermore, Berger & Johnson (1976) showed that the rate of incorporation of radiolabelled dTTP into high molecular weight material in permeabilized cells is much greater than in intact cells. The same group (Berger et al., 1978a,c) also obtained contradictory results with permeabilized PHA-stimulated normal human lymphocytes versus permeabilized mouse L cells. In lymphocytes poly(ADPR) synthetase activity increases with increase in DNA synthesis whereas in L cells poly(ADPR) synthesis is high when DNA synthesis is low. Thus, it appears that the permeabilized cell system may still be too far removed from the in vivo situation to reflect the true function(s) of ADP-ribosylation.

If the physiological function of ADP-ribosylation is to be determined the isolation and identification of individual proteins which are ADP-ribosylated in vivo is necessary. Various workers (Ueda et al., 1975; Smith & Stocken, 1973, 1975) have demonstrated that certain well characterized proteins, particularly histone H1, are ADP-ribosylated in vivo. However, such an approach is limited in that only those proteins which are easily isolated may be studied; a procedure which will quantitatively isolate all ADP-ribosylated proteins is really required. Okayama et al. (1978) and Adamietz et al.

(1979), using isolated nuclei, have developed very similar methods for the specific isolation of total ADP-ribosylated proteins by employing boronate columns which retain cis-diol containing compounds. These methods are limited, however, in that the isolation takes place at pH 8.2, a value at which a significant quantity of ADPR is released from protein (Nishizuka et al., 1969, Stone, personal communication).

The isolation method developed here, using [^3H]-adenosine-labelled mouse L1210 cells, exploited the difference in buoyant densities between RNA and DNA, and protein on caesium chloride density gradient centrifugation. Since poly(ADPR) is covalently attached to proteins the procedure potentially provides a simple and non-selective means by which to isolate proteins ADP-ribosylated in vivo. The method is, in fact, a modification of that of Rickwood et al. (1977), who used it to isolate and subsequently demonstrate the existence of free poly(ADPR) in vitro.

3.2 GROWTH CYCLE OF L1210 CELLS

Mouse L1210 cells are cultured lymphoma cells from the ascitic fluid of a DBA/2 mouse (Hutchinson et al., 1966). The advantages of using such cells are that they grow rapidly and are simple to maintain. In this way potentially few errors are introduced into the system. In culture animal cells display a growth pattern very similar to that of micro-organisms. In particular they exhibit classical growth kinetics. When cells are taken

from a stationary culture and diluted there is at first a lag phase of some hours to some days. This may be a result of accumulation of intermediary metabolites in intracellular pools which have become exhausted or perhaps the time required for readaptation of some enzymes. Growth then proceeds with the cells doubling every 12-20 hours for fast growing cells. This stage is called the logarithmic phase and during this time the cell population increases according to the formula

$$N = N_0 2^{kt}$$

$$\text{i.e. } \log N = \log N_0 + kt \log 2$$

where N_0 = initial inoculum, N = cell number at t hours and k is a regression constant.

The mean generation time, T , is the inverse of k
i.e. $T = 1/k$.

At a certain density of cells the maximum population is reached and the cells enter stationary phase. This may be a result of either cell density, exhaustion of a nutrient, accumulation of toxic products (notably hydrogen ions) or, more likely, a combination of all of these.

The metabolism and composition of cultured cells may vary considerably from one growth phase to another. During the logarithmic phase RNA, DNA and protein values increase at the same steady rate until the maximum population is approached. At this point these synthetic processes slow down, RNA decreasing first rapidly followed by protein. Of particular interest are the observations of Stone & Shall (1975). These workers showed that the activity of

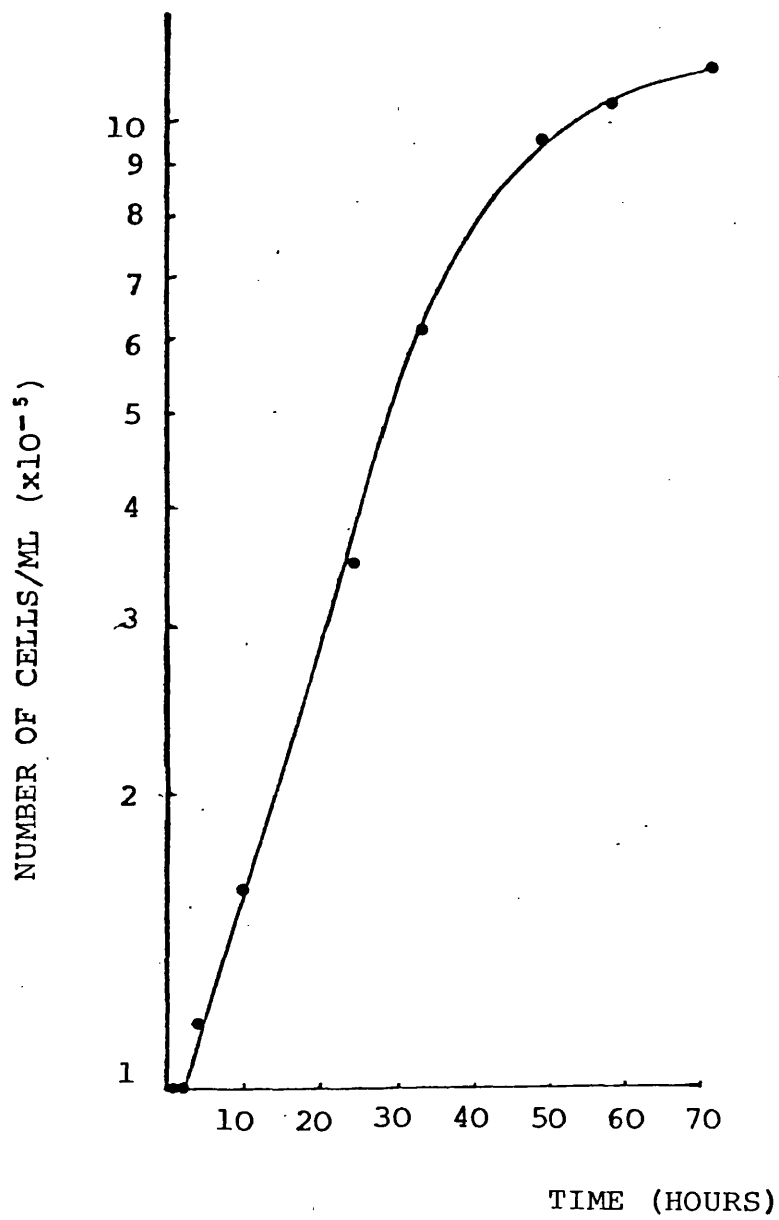
poly(ADPR) synthetase in isolated nuclei of mouse fibroblast (LS) cells increases three-fold during the growth cycle but upon dilution drops three-fold, over 12-24 hours, and then increases again with growth. More recently, Berger et al. (1978b) showed that poly(ADPR) synthetase activity in mouse L cells reaches its maximum level at early stationary phase.

The growth cycle of L1210 cells in RPMI 1640 medium is shown in Fig. 3.2. Cells were counted over a period of three days and the increase in cell number with time plotted on semilog paper. Cell viability during this time was always greater than 98%. The lag phase under the incubation conditions was 2-3 hours and the doubling time was approximately 12 hours. Stationary phase was reached at a density of about 1.2×10^6 cells/ml. Since a lag phase did occur when cells were taken from high to low density, cells were always subcultured at least 16 hours before use, in case poly(ADPR) synthesis was affected during or after this period. In fact, Morioka et al. (1980), using Friend erythroleukemic cells, showed that a medium change during the logarithmic phase effected a prolongation in the rise of poly(ADPR) synthesis during the growth cycle. Furthermore, since Stone & Shall (1975) showed that poly(ADPR) synthesis varies during the growth cycle, cells were always taken at the same density - 2×10^5 /ml, unless otherwise specified.

Fig. 3.2 Growth Curve of L1210 Cells in
RPMI 1640 Medium

L1210 cells from high density were subcultured to a density of $1 \times 10^5/\text{ml}$ and 5ml cultures incubated at 37°C in McCartney bottles. Aliquots of the cell suspension were taken twice a day and counted as described in Section 2.2.1. Each point is an average of three counts.

Fig. 3.2



3.3. PROCESSING OF MATERIAL FOR CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

In order that the analysis of putative ADP-ribosylated proteins, after their isolation on CsCl density gradients was not complicated by radiolabelled NAD^+ , AMP etc., it was felt that the removal of such acid-soluble material before centrifugation would be advantageous. Since the incorporation of $[\text{}^3\text{H}]\text{NAD}^+$ into nuclei and subsequently poly(ADPR) is a well characterized system, free of the many problems associated with in vivo systems, it was decided to develop a procedure for removal of acid-soluble material using pig thymus nuclei which could then be adapted for L1210 cells.

3.3.1 Charcoal Column

NAD^+ and poly(ADPR) are aromatic by virtue of the groups they contain, both contain adenine and NAD^+ also contains nicotinamide. Charcoal, a non-polar adsorbent, has a marked affinity for aromatic compounds. It was therefore decided to employ a charcoal column to try to selectively free ADP-ribosylated proteins from their precursor, NAD^+ . The separation was predicted to occur on the basis of the differing electron properties of the two molecules; NAD^+ contains two aromatic groups compared to poly(ADPR) which contains only one. Furthermore, the amount of NAD^+ present is likely to be significantly greater than poly(ADPR). Therefore NAD^+ may compete more effectively for binding sites on the column than protein-bound poly(ADPR). $[\text{}^3\text{H}]\text{NAD}^+$ -labelled nuclei were

applied to a charcoal column and eluted in 4M urea. The results shown in Table 3.3.1 demonstrate that some, but not all, acid-insoluble radioactivity was eluted from the column. An approximate estimate of the percentage acid-soluble material eluted or retained on the column was obtained by subtracting acid-insoluble counts from total counts. 71% of total radioactivity, 94% of acid-insoluble and 55% of acid-soluble material was bound to the column. About 50% of protein was also bound. In an attempt to release the acid-insoluble material bound to the column 0.5ml 100mM nicotinamide was applied to the column and elution carried out as described above. The results shown in Table 3.3.1 demonstrate that very little material was eluted from the column under these conditions. It appeared that under the conditions employed any material of an aromatic nature, including aromatic amino acids in protein, may have bound to the column. Thus, charcoal did not appear to selectively bind NAD^+ . It may be that by altering the conditions a greater percentage of NAD^+ may be bound and ADP-ribosylated protein eluted. However, since the method did not seem to be reliable or reproducible it was consequently abandoned in favour of a simpler approach.

3.3.2 Biogel P10 Chromatography

One difference between NAD^+ and poly(ADPR) which may be easily exploited to achieve a simple separation of the two molecules is their size. Consequently, separation was attempted by chromatography on Biogel P10. The

Table 3.3.1 Efficiency of Charcoal as a Means of Separating [³H]ADP-Ribosylated Proteins
from their Precursor, [³H]NAD⁺

	Quantity applied to column	Quantity eluted from column	Quantity bound to column	Quantity eluted from column with Nicotinamide
Total c.p.m.	23,507 ± 1,276.6	7,993 ± 150.7	15,514 ± 1,216.0	89 ± 4.5
Acid-insoluble c.p.m.	6,799 ± 320.1	400 ± 4.6	6,399 ± 323.6	97 ± 2.1
Protein (μg)	47.3 ± 1.51	23.3 ± 3.9	24.0 ± 3.9	0

[³H]NAD⁺-labelled pig thymus nuclei were applied to a ground charcoal column of dimensions 5cm x 1.75cm² and the material eluted with 2.5ml 4M urea at room temperature. 100μl samples were counted for both total and acid-insoluble (GF/C discs) radioactivity as described in Section 2.2.3. Protein quantities were determined as described in Section 2.2.14. Each value is the mean of five samples and the error is represented as the standard error of that mean (S.E.M.).

column was calibrated as described in Section 2.2.7. The void volume was estimated as 10ml. [^3H]NAD $^+$ -labelled nuclei were applied to the column and eluted in 4M urea, 0.1M NaAc/HAc - pH 6.5. Under these conditions the protein-ADPR bond is stable (Nishizuka et al., 1969). A representative profile of the radioactivity eluted during chromatography is shown in Fig. 3.3.2. A good separation was achieved between acid-insoluble material, which eluted just after the void volume of the column, and acid-soluble material. Acid-insoluble material accounted for about 20% of the radioactivity on the column. As controls DNA, RNA and protein were labelled overnight with [^3H]thymidine, [^3H]uridine and [^3H]leucine, respectively, and also applied to the column. In addition, [^3H]NAD $^+$ alone and [^3H]NAD $^+$ -labelled nuclei in the presence of 2mM 3-aminobenzamide (3-AB), a potent inhibitor of poly(ADPR) synthetase (see Section 1.3), were applied. The profiles obtained with [^3H]thymidine, [^3H]uridine and [^3H]leucine-labelled cells were very similar to that with [^3H]NAD $^+$ -labelled nuclei. No acid-insoluble peak of radioactivity was observed when [^3H]NAD $^+$ alone or [^3H]NAD $^+$ -labelled nuclei in the presence of 3-AB were applied. The results and recoveries obtained during these experiments are summarized in Table 3.3.2; Biogel P10 chromatography provided a good means by which to separate ADP-ribosylated proteins from acid-soluble material.

The procedure was subsequently adopted for mouse L1210 cells labelled with [^3H]adenosine for 18h at 40 $\mu\text{Ci}/\text{ml}$. Cells were washed by centrifuging in 0.15M NaCl and

Fig. 3.3.2 Biogel P10 Fractionation of [^3H]NAD $^+$ -
Labelled Nuclei

0.5ml [^3H]NAD $^+$ -labelled pig thymus nuclei were applied to a Biogel P10 column of dimensions 25cm x 3.14cm 2 and eluted in 4M urea, 0.1M NaAc/HAc-pH 6.5 at a rate of 20ml/h. Chromatography took place at room temperature. 2ml fractions were collected and total and acid-insoluble (GF/C discs) radioactivity determined as described in Section 2.2.3. \square Total radioactivity, \blacktriangle acid-insoluble radioactivity, A - Blue Dextran, B - bovine serum albumin, C - cytochrome c, D - phenol red.

Fig. 3.3.2

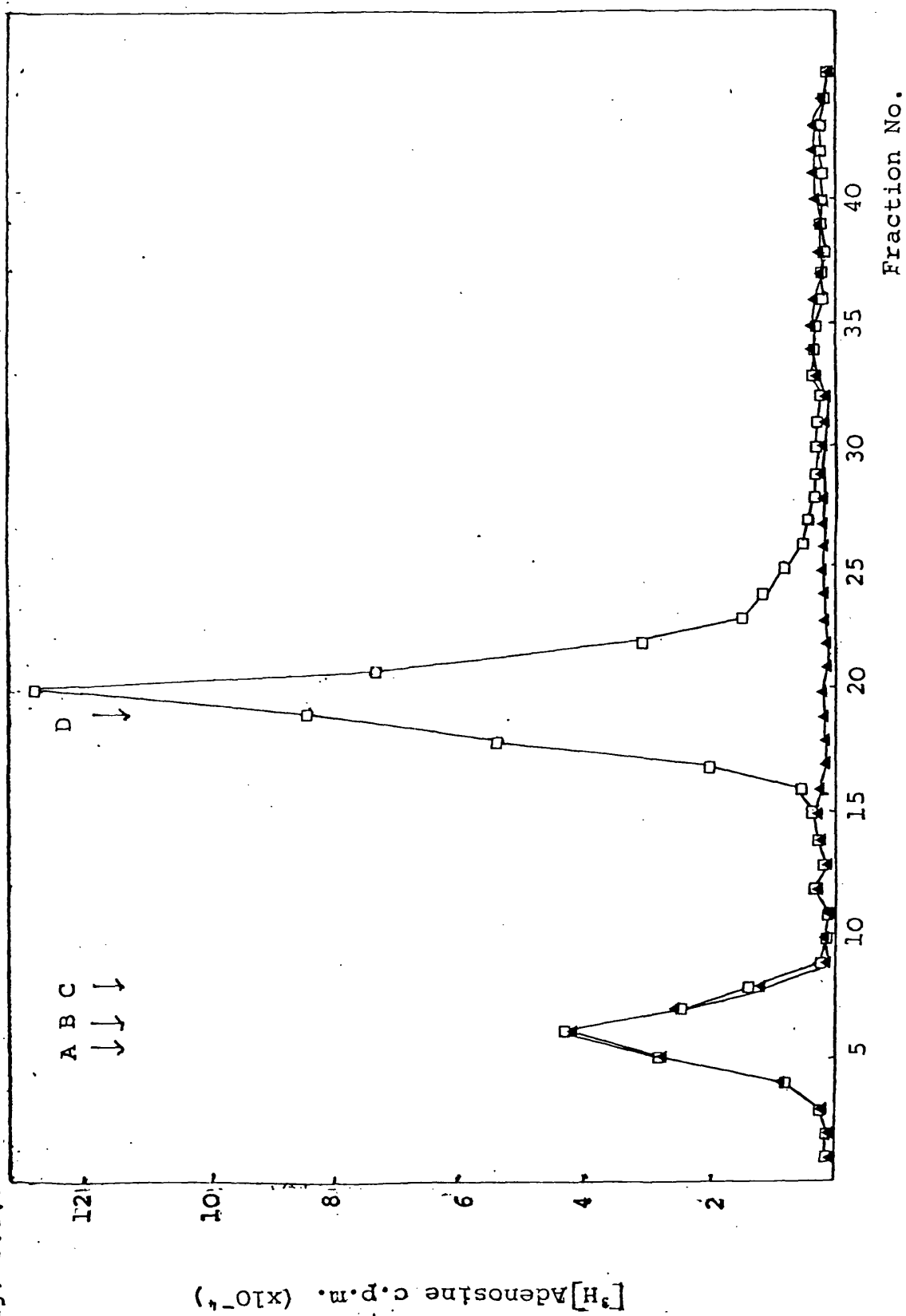


Table 3.3.2 Biogel P10 Fractionation of [^3H]NAD $^+$ -Labelled Nuclei

	c.p.m. Added	c.p.m. Recovered	% Recovered	c.p.m. Acid- Soluble	% Acid- Soluble	c.p.m. Acid- Insoluble	% Acid- Insoluble
[^3H]NAD $^+$ + nuclei	6.60×10^5	5.61×10^5	85.0	4.34×10^5	77.7	1.23×10^5	22.0
[^3H]NAD $^+$ alone	6.63×10^5	5.87×10^5	88.5	5.66×10^5	96.5	5.87×10^2	0.1
[^3H]NAD $^+$ + nuclei + 2mM 3-AB	6.59×10^5	5.73×10^5	87.0	5.36×10^5	93.5	6.88×10^3	1.2
[^3H]Thymidine + cells	1.35×10^6	1.14×10^6	87.0	9.35×10^5	82.0	1.87×10^5	16.4
[^3H]Uridine + cells	1.34×10^6	1.14×10^6	85.0	9.50×10^5	83.0	1.77×10^5	15.5
[^3H]Leucine + cells	1.32×10^6	1.14×10^6	86.0	9.40×10^5	82.5	2.00×10^5	17.8

[^3H]NAD $^+$ -labelled pig thymus nuclei were fractionated on Biogel P10 as described in Section 2.2.7. [^3H]NAD $^+$ alone and [^3H]NAD $^+$ -labelled nuclei in the presence of 2mM 3-AB were also fractionated as controls, as were cells incubated with 2 μCi of [^3H]thymidine, [^3H]uridine and [^3H]leucine, overnight. Each value is an average of 2 experiments.

the final pellet dissolved in 0.5ml 4M urea, 0.1M NaAc/HAc-pH 6.5 before chromatography. Separation and recovery obtained were very similar to that using isolated nuclei. Under the conditions employed about 10% of radioactivity incorporated was as acid-insoluble material (2.3×10^6 c.p.m./5ml). However, by harvesting cells in 0.15M NaCl about 11% of acid-insoluble radioactivity was lost into the supernatant on centrifugation. Furthermore, this loss was not much reduced on prolonged centrifugation. Centrifuging the cells in the absence of NaCl still brought about a significant loss of acid-insoluble radioactivity into the supernatant (see Table 3.3.3). Clearly a method was required which would allow a quantitative recovery of acid-insoluble radioactivity. Furthermore, in the presence of 0.15M NaCl some nuclear enzymes, of particular relevance poly(ADPR) glycohydrolase, may have been released from DNA and subsequently activated. On isolation, ADP-ribosylated proteins may consequently exhibit characteristics which did not exist in vivo. Thus, a procedure was required which would immediately inactivate the cell's enzymes and also allow a quantitative recovery of acid-insoluble radioactivity. The development of such methods is described below.

3.3.3 Improved Techniques for Isolation of Acid-Insoluble Material

Three reagents capable of inactivating the cell's enzymes and of precipitating high molecular weight material were chosen. These were ethanol, acetone and TCA. Ethanol and acetone solutions were buffered with 50mM

Table 3.3.3 Processing of [³H]Adenosine-Labelled Cells - Efficiency of Various Treatments in Retaining Acid-Insoluble Radioactivity

Method	Total c.p.m. in Supernatant	Acid-insoluble c.p.m. in Supernatant	% Total c.p.m. in Supernatant	% Acid-insoluble c.p.m. in Supernatant
0.15M NaCl 10 min 3,000g	1.05 x 10 ⁸ ±	2.97 x 10 ⁵ ±	79.5	11.0
	1.10 x 10 ⁶	1.36 x 10 ⁴		
5 min 300g	1.03 x 10 ⁸ ±	3.05 x 10 ⁵ ±	78.0	12.2
	1.04 x 10 ⁶	1.59 x 10 ⁴		
10 min 300g	1.03 x 10 ⁸ ±	1.52 x 10 ⁵ ±	78.0	6.1
	1.15 x 10 ⁶	2.04 x 10 ⁴		
EtOH 20 min 800g	3.20 x 10 ⁷ ±	2.75 x 10 ⁵ ±	24.2	11.0
	1.92 x 10 ⁵	1.10 x 10 ⁴		
Acetone 20 min 800g	3.05 x 10 ⁷ ±	8.05 x 10 ⁴ ±	23.2	3.2
	2.85 x 10 ⁵	4.80 x 10 ³		
TCA 20 min 800g	3.00 x 10 ⁷ ±	7.50 x 10 ⁴ ±	22.7	3.0
	2.64 x 10 ⁵	4.11 x 10 ³		
TCA/Acetone 20 min 800g	3.10 x 10 ⁷ ±	7.60 x 10 ⁴ ±	23.5	3.0
	2.75 x 10 ⁵	5.08 x 10 ³		

Cells (5ml) were centrifuged at 40C. Each value is the mean ± S.E.M. of 6 samples.

NaAc/HAc ~ pH5 so that the protein-ADPR bond was kept intact. After labelling, 90% of the medium was carefully aspirated off and the cells immediately frozen in liquid N₂ to inactivate any degradative enzymes. All solutions were added before the cells were allowed to thaw and the mixture then left on ice for one hour to allow for precipitation. Precipitation was brought about by either 70% (v/v) ethanol; 70% (v/v) acetone or 20% (w/v) TCA. The resultant precipitate was then processed in a number of ways: (i) centrifuged twice in 70% (v/v) EtOH, then twice in 60% (v/v) EtOH, (ii) centrifuged twice in 70% (v/v) acetone, then twice in 60% (v/v) acetone, (iii) centrifuged three times in 20% (w/v) TCA, (iv) centrifuged twice in 20% (w/v) TCA, then twice in 70% (v/v) acetone. The total losses incurred during these treatments are shown in Table 3.3.3.

Ethanol appeared to allow the release of acid-insoluble material into the supernatant to a greater extent than either acetone or TCA. Dick & Johns (1968) demonstrated that the more hydrophobic histone fractions, predominantly the arginine-rich histones H3 and H4, were selectively extracted in ethanol/acetic acid. Some of the acid-insoluble radioactivity lost during the ethanol washes may therefore be attributed to this phenomenon. Similarly, other hydrophobic proteins may also have been lost.

Precipitating cells in 20% (w/v) TCA and then washing them free of acid in 70% (v/v) acetone proved to be the most efficient means by which to both remove acid-soluble material and retain acid-insoluble material; 0.1% of acid-

soluble radioactivity was retained in the pellet and 3% of acid-insoluble radioactivity was lost to the supernatant. Thus, about 5% of counts applied to the gradient were acid-soluble. This procedure was therefore adopted for all further experiments.

3.4 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

When a sample of mixed macromolecular population is centrifuged in a solution of CsCl two processes occur. First, a concentration gradient of CsCl is formed, the slope and range of which depends on the speed and temperature of the run and on the concentration of salt used. Secondly, the macromolecules within the solution approach an equilibrium state at which point the rate of sedimentation in one direction is equal to the rate of diffusion in the opposite direction. Each species will band within the concentration gradient formed at a level equal to its own buoyant density.

The choice of CsCl was made because the high solubility and high density of the salt enabled a good separation of proteins and nucleic acids, the buoyant density of nucleic acids in CsCl is high. In addition, CsCl solution does not quench liquid scintillation fluids as does, for example, Cs_2SO_4 . The resolution obtained in CsCl is also greater than in Cs_2SO_4 . The initial density of CsCl was chosen to produce, on centrifugation, a density range which would allow maximum separation of proteins and nucleic acids. Centrifugation of a CsCl solution of initial density 1.55 (50% (w/v) soln.) in a Beckman SW50.1

rotor at 200,000g and 15°C was predicted (Birnie, 1978) to result in a gradient of a density ranging from 1.25 to 1.9. Under these conditions proteins should band at the top of the gradient and RNA and DNA at the bottom. A temperature of 15°C rather than 4°C was employed since this allowed a greater density range to be used without re-crystallization of CsCl.

The ionic interaction between proteins and DNA or RNA is sometimes very strong and, furthermore, protein-bound poly(ADPR) has a tendency to be insoluble in solution (possibly as a result of aggregation) (Adamietz et al., 1975). Consequently, 5M urea was present during centrifugation so that these bonds were dissociated. Initially, the sample was added to a solution of 5M urea, 50% (w/v) CsCl. However, on centrifugation, analysis of the distribution of radioactivity revealed that the bands were not as sharply defined as expected (see Fig. 3.4.1). It seemed that the dissociation of macromolecular species might still have been incomplete. Subsequently, the sample was first dissolved in 2.5ml 10M urea, left for 15 minutes at 15°C and then made up to 5ml with 100% (w/v) CsCl. The solution was then mixed thoroughly and left for a further 15 minutes before centrifugation. The distribution of radioactivity shown in Fig. 3.4.2B demonstrates that a greater degree of solubilization and denaturation occurred under these conditions. This procedure was therefore adopted for all further centrifugations. The sample was buffered at pH5 with NaAc/HAc to ensure that protein-ADPR bonds were kept intact. Furthermore, phenylmethanesulphonyl

Fig. 3.4.1. Caesium Chloride Density Gradient
Centrifugation - Attempted Separation
of Protein from DNA and RNA

A 5ml culture of mouse L1210 cells, labelled with 40 μ Ci/ml of [3 H]adenosine for 18h, was washed as described in Section 2.2.9, then dissolved in 5ml 5M urea, 50% (w/v) CsCl, 0.1M NaAc/HAc - pH5, 0.1mM PMSF. The material was then centrifuged for 72h as described in Section 2.2.10 and acid-insoluble radioactivity (on paper discs) counted as described in Section 2.2.3. Density was determined as described in Section 2.2.10. -O- [3 H]adenosine, --- density.

Fig. 3.4.1

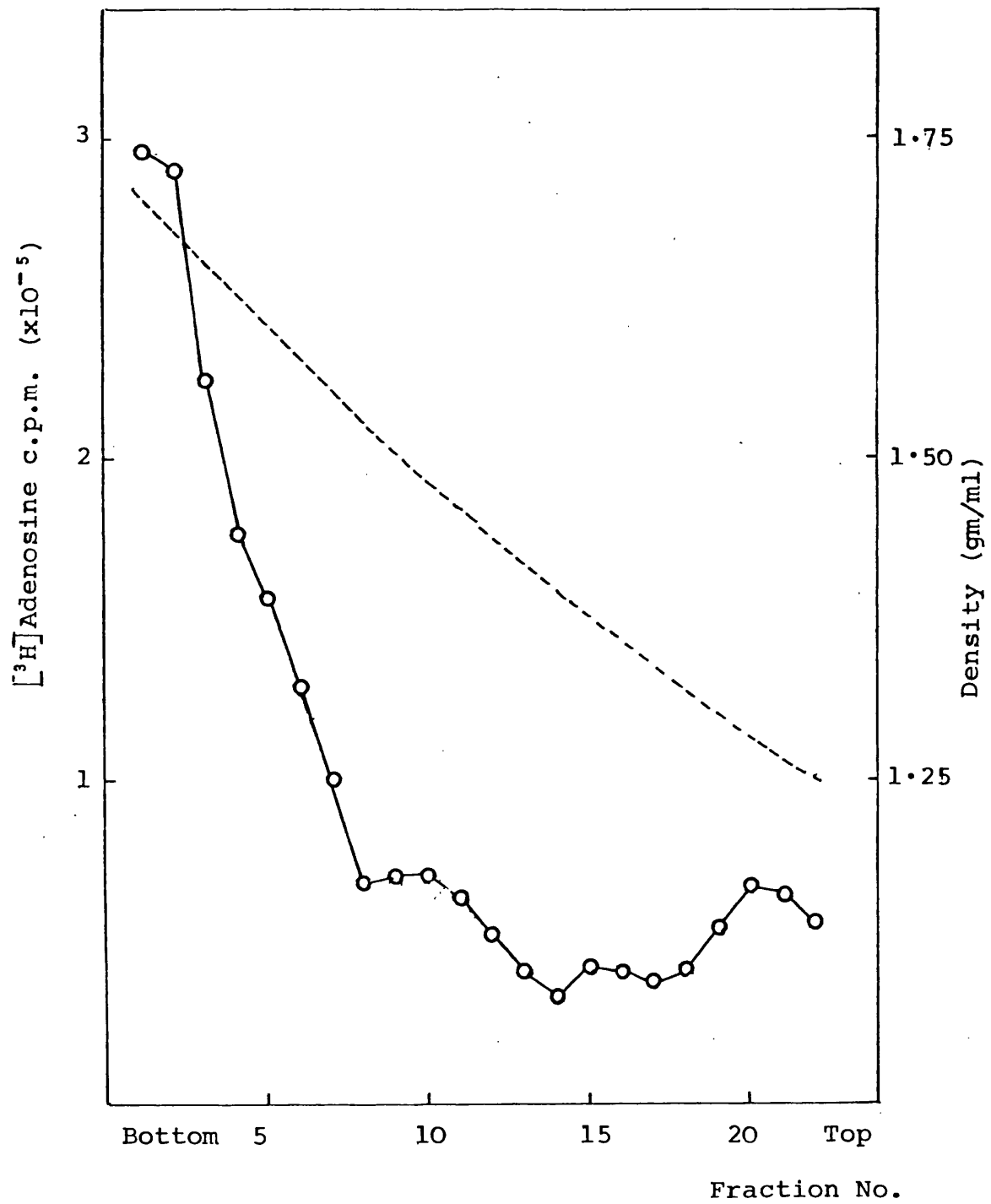


Fig. 3.4.2 Caesium Chloride Density Gradient
 Centrifugation - Separation of DNA
 and RNA from Protein

5ml cultures of mouse L1210 cells labelled with 40 μ Ci/ml of either [3 H]adenosine, [3 H]thymidine, [3 H]uridine or [3 H]leucine for 18h were washed as described in Section 2.2.9 and the material centrifuged in CsCl/urea for 72h as described in Section 2.2.10. Acid-insoluble radioactivity (on paper discs) was counted as described in Section 2.2.3. For [3 H]adenosine 5ml of cells were applied to the gradient and for [3 H]thymidine, [3 H]uridine and [3 H]leucine 10ml of cells were applied. A: \bullet [3 H]thymidine, \square [3 H]uridine, \blacksquare [3 H]leucine. B: \blacksquare [3 H]adenosine, ---- density.

Fig. 3.4.2A

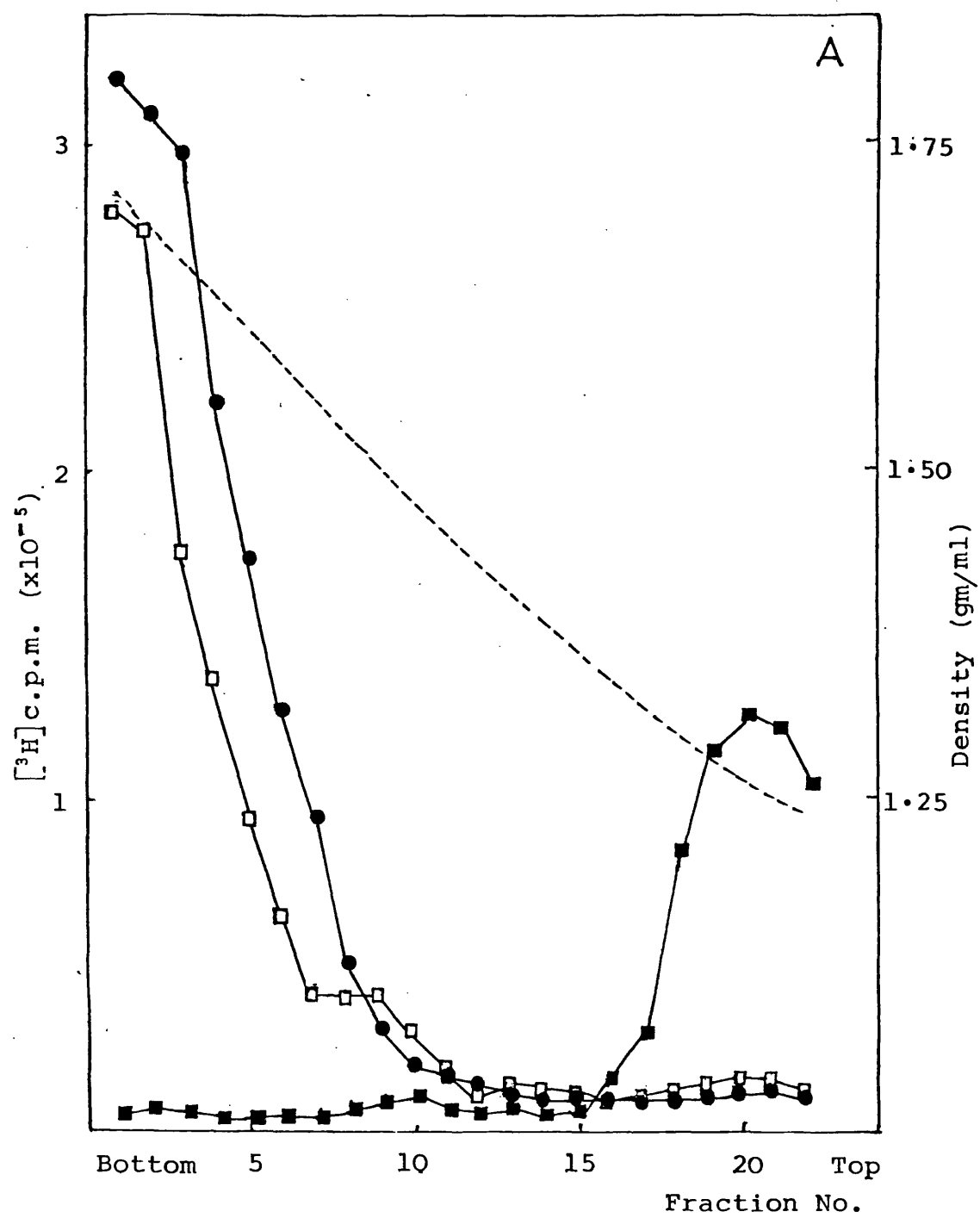
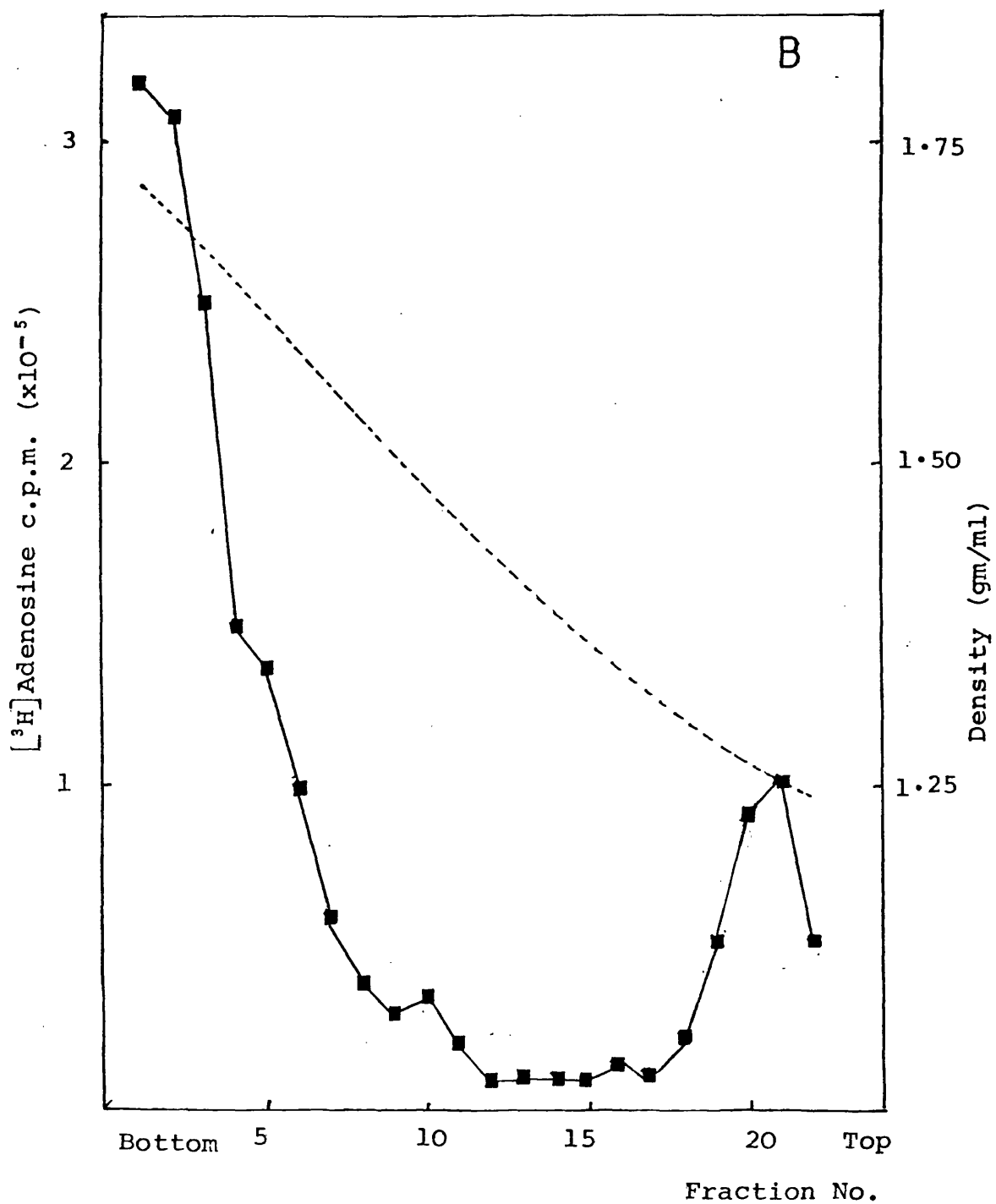


Fig. 3.4.2B



fluoride (PMSF) was present in all samples during centrifugation to prevent proteolysis and the resultant decrease in resolution that this might have produced. Ultracentrifugation under these conditions was found to give greatest resolution and recovery (see Table 3.4 for recovery). 13% of acid-insoluble radioactivity banded at the top of the gradient.

Initially, cells were labelled with [^3H]adenosine and centrifuged for different lengths of time to determine the duration of centrifugation required to allow good separation between the macromolecular species present. Fig. 3.4.3 shows that good separation between the two peaks of radioactivity present is only obtained after a 72 hour centrifugation. At this point the basal level of radioactivity between the two peaks reached it lowest level. This suggested that at shorter periods of centrifugation the macromolecular species might still have been 'mixed' and only on prolonged centrifugation did they separate and approach their buoyant densities. This degree of resolution was obtained on centrifuging 5 or 10ml samples of cells per tube. However, on centrifuging 20 or 30ml of cells the resolution was much decreased. As pointed out by Birnie (1978), the capacity of a gradient is often much less than that predicted. Subsequently, for all further experiments (unless otherwise specified) 5ml samples of cells were routinely used per centrifuge tube.

Cells were then labelled for 18h with [^3H]thymidine, [^3H]uridine and [^3H]leucine to label DNA, RNA and protein,

Table 3.4 Recovery of [³H] of Label during Isolation of Macromolecular Species

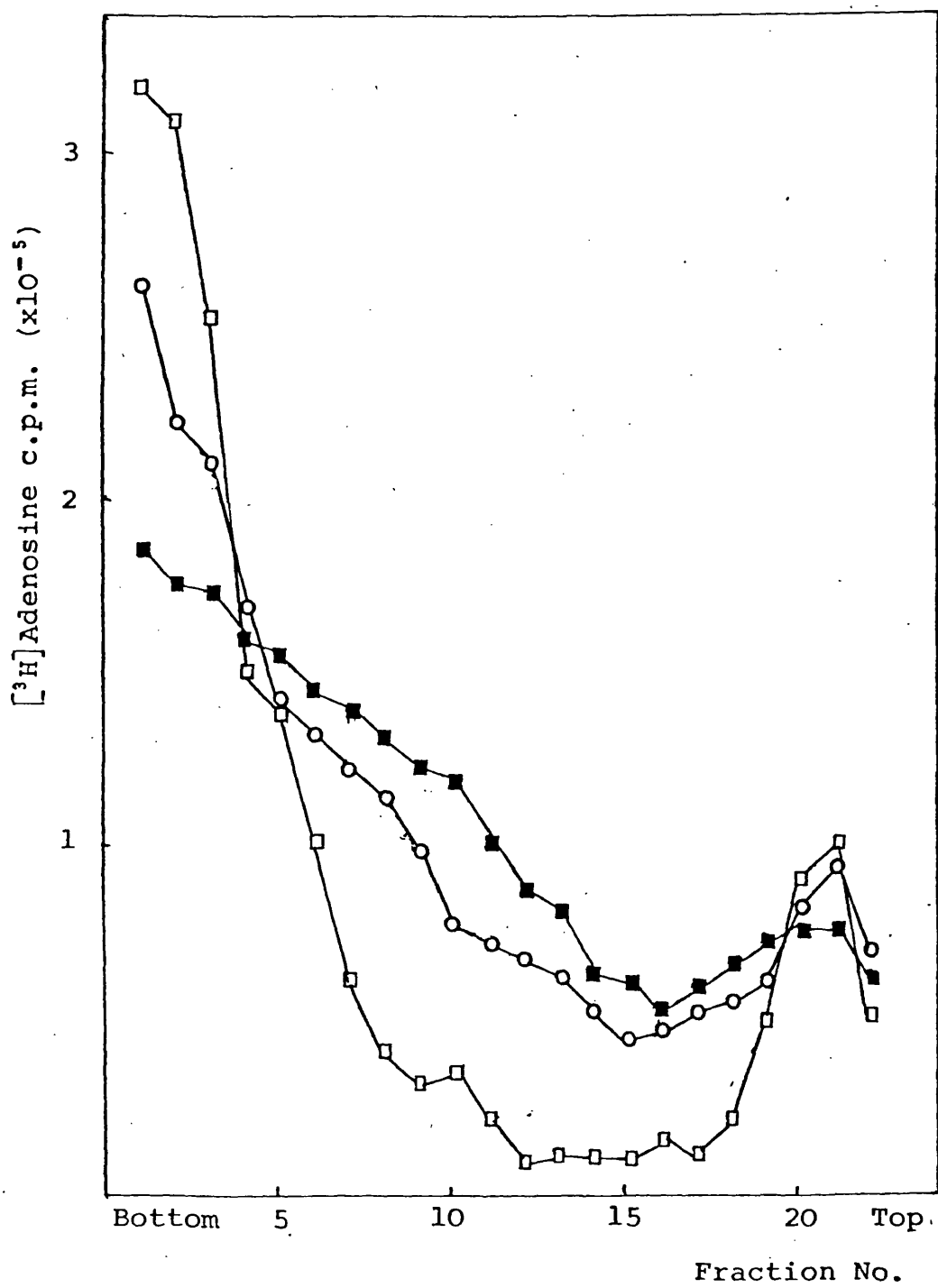
Label	Total Radio-activity Added to Cells (c.p.m.)	Total Radio-activity Incorporated	Acid-insoluble Radioactivity Incorporated	Acid-insoluble Radioactivity Incorporated After First Wash (Step 1)	Acid-insoluble Radioactivity Incorporated After Centrifugation (Step 2)	Acid-insoluble Radioactivity Incorporated After Second Wash (Step 3)*
[³ H] Adenosine	1.32 x 10 ⁸ + 6.10 x 10 ⁵ (100%)	2.72 x 10 ⁷ + 1.50 x 10 ⁵ (20.6%)	2.33 x 10 ⁶ + 5.20 x 10 ³ (1.77%)	2.26 x 10 ⁶ + 4.90 x 10 ³ (1.71%)	2.25 x 10 ⁶ + 4.50 x 10 ³ (1.70%)	2.78 x 10 ⁵ + 4.80 x 10 ² (0.21%)
[³ H] Thymidine	1.31 x 10 ⁸ + 5.82 x 10 ⁵ (100%)	2.77 x 10 ⁷ + 2.05 x 10 ⁵ (21.0%)	1.08 x 10 ⁶ + 5.10 x 10 ³ (0.82%)	1.05 x 10 ⁶ + 5.30 x 10 ³ (0.80%)	1.04 x 10 ⁶ + 5.20 x 10 ³ (0.79%)	2.08 x 10 ⁴ + 0.64 x 10 ² (0.02%)
[³ H] Uridine	1.32 x 10 ⁸ + 6.05 x 10 ⁵ (100%)	2.71 x 10 ⁷ + 1.82 x 10 ⁵ (20.5%)	0.78 x 10 ⁶ + 6.30 x 10 ³ (0.59%)	0.76 x 10 ⁶ + 6.20 x 10 ³ (0.58%)	0.75 x 10 ⁶ + 5.60 x 10 ³ (0.57%)	2.25 x 10 ⁴ + 0.61 x 10 ² (0.02%)
[³ H] Leucine	1.31 x 10 ⁸ + 6.15 x 10 ⁵ (100%)	2.75 x 10 ⁷ + 1.73 x 10 ⁵ (21%)	0.67 x 10 ⁶ + 5.70 x 10 ³ (0.51%)	0.64 x 10 ⁶ + 5.60 x 10 ³ (0.49%)	0.64 x 10 ⁶ + 4.80 x 10 ³ (0.49%)	0.61 x 10 ⁶ + 4.20 x 10 ³ (0.47%)
* The analysis in Step 3 was carried out using only the top 1.5ml of the gradient						

Mouse L1210 cells (5ml) were labelled for 18h with 200μCi of either [³H]adenosine, [³H]thymidine, [³H]uridine or [³H]leucine, washed (Step 1; Section 2.2.9), centrifuged (Step 2; Section 2.2.10) and washed again (Step 3; Section 2.2.11). Acid-insoluble counts (precipitated on paper discs) and total counts were determined as described in Section 2.2.3. The table compares the recoveries obtained at each stage of the isolation procedure. Values are expressed as mean ± S.E.M. of 3 samples.

Fig. 3.4.3 Caesium Chloride Density Gradient
Centrifugation - Distribution of
Labelled Macromolecules with Time

5ml of mouse L1210 cells were labelled for 18h with [^3H] adenosine at 40 $\mu\text{Ci/ml}$ and washed as described in Section 2.2.9. The acid-insoluble material obtained was then centrifuged, as described in Section 2.2.10, for different lengths of time. Acid-insoluble radioactivity (on paper discs) was counted as described in Section 2.2.3. —■— 24h centrifugation, —○— 48h centrifugation, —□— 72h centrifugation.

Fig. 3.4.3



respectively. In this way the position of these macromolecules on the gradient could be determined and related to that obtained with [^3H]adenosine-labelled cells. The results of this experiment are shown in Fig. 3.4.2A. 93% of [^3H]uridine and 96% of [^3H]thymidine banded at the bottom of the gradient and 96% of [^3H]leucine banded at the top of the gradient. Thus, RNA and DNA banded at the bottom of the gradient whereas protein banded at the top. [^3H]adenosine-labelled material in association with protein at the top of the gradient suggested that this might be putative ADP-ribosylated protein. This material accounted for about 13% of acid-insoluble radioactivity on the gradient. The density profile obtained was very similar to that predicted, suggesting that the equilibrium gradient had formed. Thus, the desired separation of putative ADP-ribosylated protein from DNA and RNA had been achieved.

3.5 DESALTING OF GRADIENT MATERIAL

So that the analysis and characterization of putative ADP-ribosylated protein from the top of the gradient might be carried out without interference from salt, it was necessary to remove the CsCl and urea from these fractions before such experiments could be commenced.

3.5.1. Dialysis

Initially, dialysis was employed because it was a standard method of removing salt. The top 1.5ml of each gradient were dialysed against 1 litre 50mM NaAc/EAc-

pH5 overnight and then for a further four hours after a change of buffer. A 50 μ l sample was counted for total and acid-insoluble (on paper discs) radioactivity before and after dialysis as described in Section 2.2.3. However, using this procedure approximately 11% of acid-insoluble counts and 9% of total counts were lost. Under the conditions employed the release of acid-soluble counts was unlikely. Thus, the loss may have been a result of precipitation of ADP-ribosylated protein, probably on to the inside wall of the dialysis tubing. If this were so then it would agree with the finding of Adamietz et al. (1975) that in the absence of urea (urea is being removed during dialysis) ADP-ribosylated protein tends to be insoluble, possibly as a result of aggregation, and subsequently precipitates out of solution. The method was therefore abandoned in favour of a more efficient and reliable procedure.

3.5.2 TCA Precipitation

This procedure was essentially the same as that adopted to remove excess label and radioactive precursors from ADP-ribosylated protein before ultracentrifugation. The top 1.5ml of each gradient were precipitated with 20% (w/v) TCA. Since the quantity of material was small, the sample was left overnight at 4°C to ensure complete precipitation. The precipitate was then washed once with TCA and twice with acetone as described in Section 3.3.3. The recovery of acid-insoluble radioactivity during this procedure was about 98% (see Table 3.4). This method was therefore adopted for all further experiments.

3.6 DETERMINATION OF ANY PROTEOLYTIC ACTIVITY DURING CsCl DENSITY GRADIENT CENTRIFUGATION

The addition of urea and PMSF to CsCl gradients should have excluded any proteolytic activity during centrifugation by denaturation or inactivation of the enzymes responsible. The profile of radioactivity obtained on centrifugation supports this contention since the band representing protein was confined to a relatively small number of fractions. A significant amount of proteolysis would have been expected to produce a broader band due to the diffusion of molecules of relatively small size. However, PMSF has only been reported to inactivate the serine proteases (Fahrney & Gold, 1968). Furthermore, the denaturation caused by urea may not have been sufficient for the inactivation of other proteolytic enzymes. Since the aim of this work was to produce a non-selective and quantitative procedure for the isolation of ADP-ribosylated protein which could be adapted for many different systems and so permit a comprehensive comparison of ADP-ribosylation in vivo and in vitro, this investigation was of paramount importance. Two different approaches were adopted to assay for proteolytic activity.

3.6.1 'Mixing' Experiment

If proteolytic enzymes were still active under the conditions employed during centrifugation, then their detection should be made possible by the release of acid-soluble radioactivity on incubation of the sample with nuclei previously labelled with $[^3\text{H}]\text{NAD}^+$. On treatment

with TCA a significant number of labelled ADP-ribosylated peptide chains from nuclei, formed as a result of proteolytic activity, were predicted to remain acid-soluble. Thus, nuclei were isolated from L1210 cells (see Section 2.2.5) and then labelled with [^3H]NAD $^+$ as described in Section 2.2.6. 0.5ml (8×10^4) nuclei were then incubated at 15°C with 2ml samples of harvested cells (8×10^5) in CsCl/urea/NaAc - pH5/PMSF. The number of cells exceeded that of nuclei by a factor of ten to ensure that the effects of proteolytic activity, if any, could be easily detected. Acid-insolubility was then observed at a number of time points after mixing. Since the concentration of CsCl at the top of the gradient decreased with time on centrifugation, the concentration at each time point decreased accordingly to reflect the situation during centrifugation. Thus, at 0 hours the concentration was 50% (w/v) and at 72 hours it was 27% (w/v) (equivalent to the estimated density of 1.25 on the top of the gradient after centrifugation). The results of this analysis are shown in Table 3.6.1. No decrease in acid-insoluble radioactivity with time was observed. These results suggest the absence of any proteolytic activity under the conditions employed during centrifugation.

3.6.2 Sephadex G-75 Chromatography

One criticism of the mixing experiment described above is that it is dependent on the formation of acid-soluble radioactivity by proteolytic action. Some enzymes

Table 3.6.1 Analysis of [^3H]NAD $^+$ -Labelled Acid-Insoluble Material from Nuclei of L1210 Cells with Time after Incubation with L1210 Cells in CsCl/Urea NaAc-pH5/PMSF

% (w/v) CsCl	Time after incubation (hours)	Acid-insoluble radioactivity (c.p.m.)
50	0	431 \pm 26
42	24	485 \pm 33
35	48	461 \pm 24
27	72	476 \pm 26

0.5ml (8×10^4) nuclei in incubation buffer were incubated at 15°C with 2ml samples of harvested cells (8×10^5) in 27-50% (w/v) CsCl, 5M urea, 0.1M NaAc/HAc-pH5, 0.1mM PMSF. 10 μ l aliquots were then assayed for acid-insoluble radioactivity (paper discs) as described in Section 2.2.3. Each value is the mean of four samples and the error is represented as the standard error of that mean (S.E.M.).

may only cleave at a relatively 'rare' amino acid in the polypeptide chain and subsequently produce peptides that are still intrinsically acid-insoluble. Even cleavage at relatively common amino acids may still produce acid-insoluble peptides. This prompted an investigation into the size distribution of the labelled ADP-ribosylated protein by Sephadex G-75 column chromatography. Two samples were analysed (five times each): the initial time point just after mixing, and the final time point corresponding to the completion of centrifugation. Again, the conditions employed during chromatography were chosen to ensure the stability of the ADPR-protein bond (pH5) and the solubility of the ADP-ribosylated protein (5M urea). Representative profiles of the distribution of radioactivity observed on elution of these samples is shown in Fig. 3.6.2 A & B. Ribonuclease (mol.wt. 13,700) was employed as a molecular weight marker. The profiles obtained for both samples were very similar. The majority of radioactivity eluted between fractions 55 and 80. The sample which had been incubated in CsCl/urea for 72 hours did, however, exhibit an additional small peak of radioactivity eluting at fractions 91-94. This peak corresponded to about 3% of the radioactivity on the column. These results confirm and extend those observed in the mixing experiment and show conclusively that negligible proteolytic activity occurred during centrifugation of the cell sample in CsCl/urea. The isolation of putative ADP-ribosylated protein by CsCl density gradient centrifugation was therefore both quantitative

Fig. 3.6.2 Sephadex G-75 Column Chromatography of
[³H]NAD⁺-Labelled L1210 Nuclei Incubated
with L1210 Cells in CsCl/Urea/NaAc-pH5/PMSF

Samples (0.5ml) were applied to a column (90cm x 1.77cm²) of Sephadex G-75, equilibrated and eluted in 5M urea, 0.1M NaAc/HAc - pH5. Elution was carried out at a flow rate of 55ml/h and 2.5ml fractions were collected. Aliquots (0.5ml) were counted for radioactivity as described in Section 2.2.3. The void volume of the column was 80ml and RNase A (mol.wt. 13,700) was used as a mol.wt. marker. The radioactivity represented is that of the total material applied.

Fig. 3.6.2A

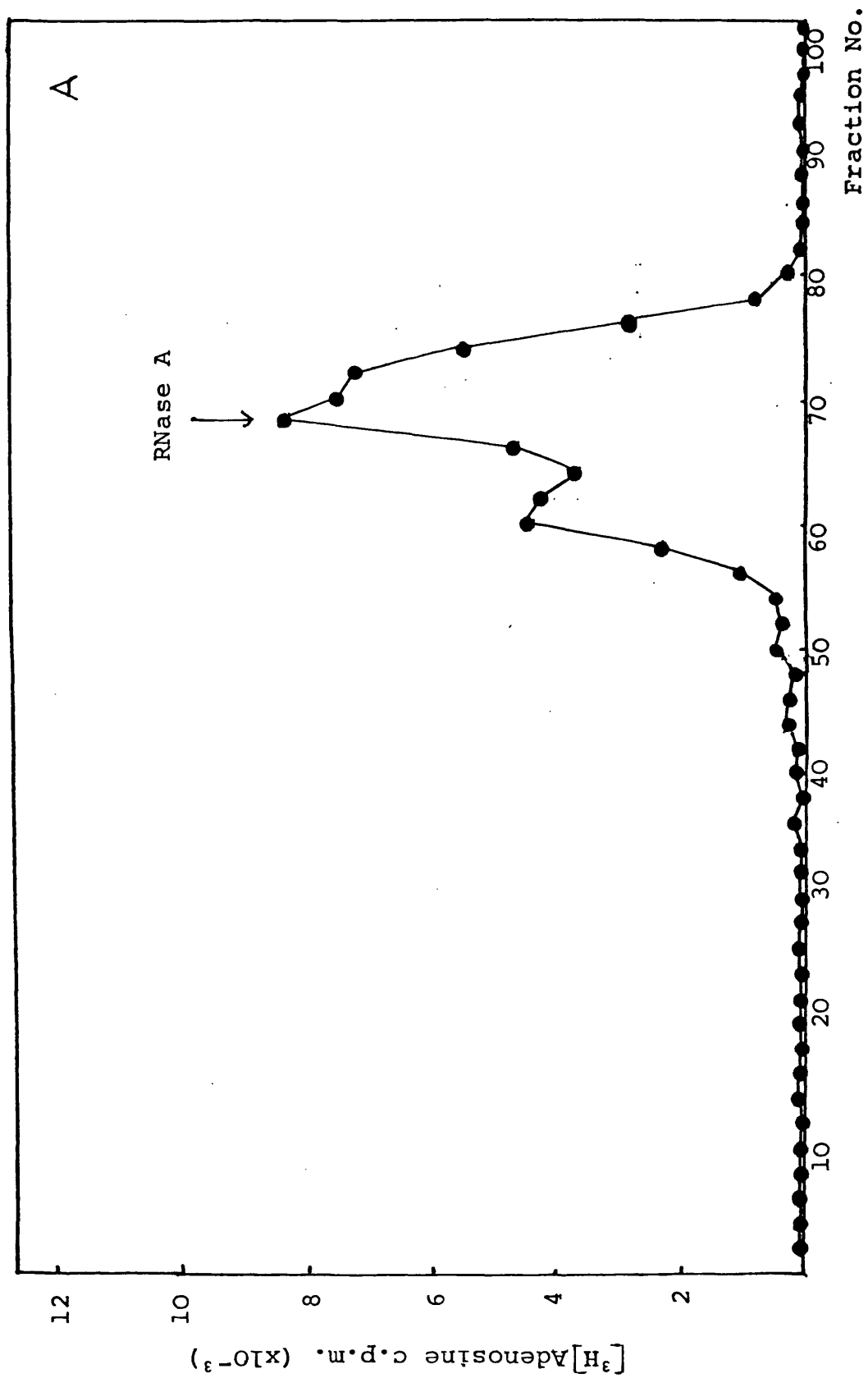
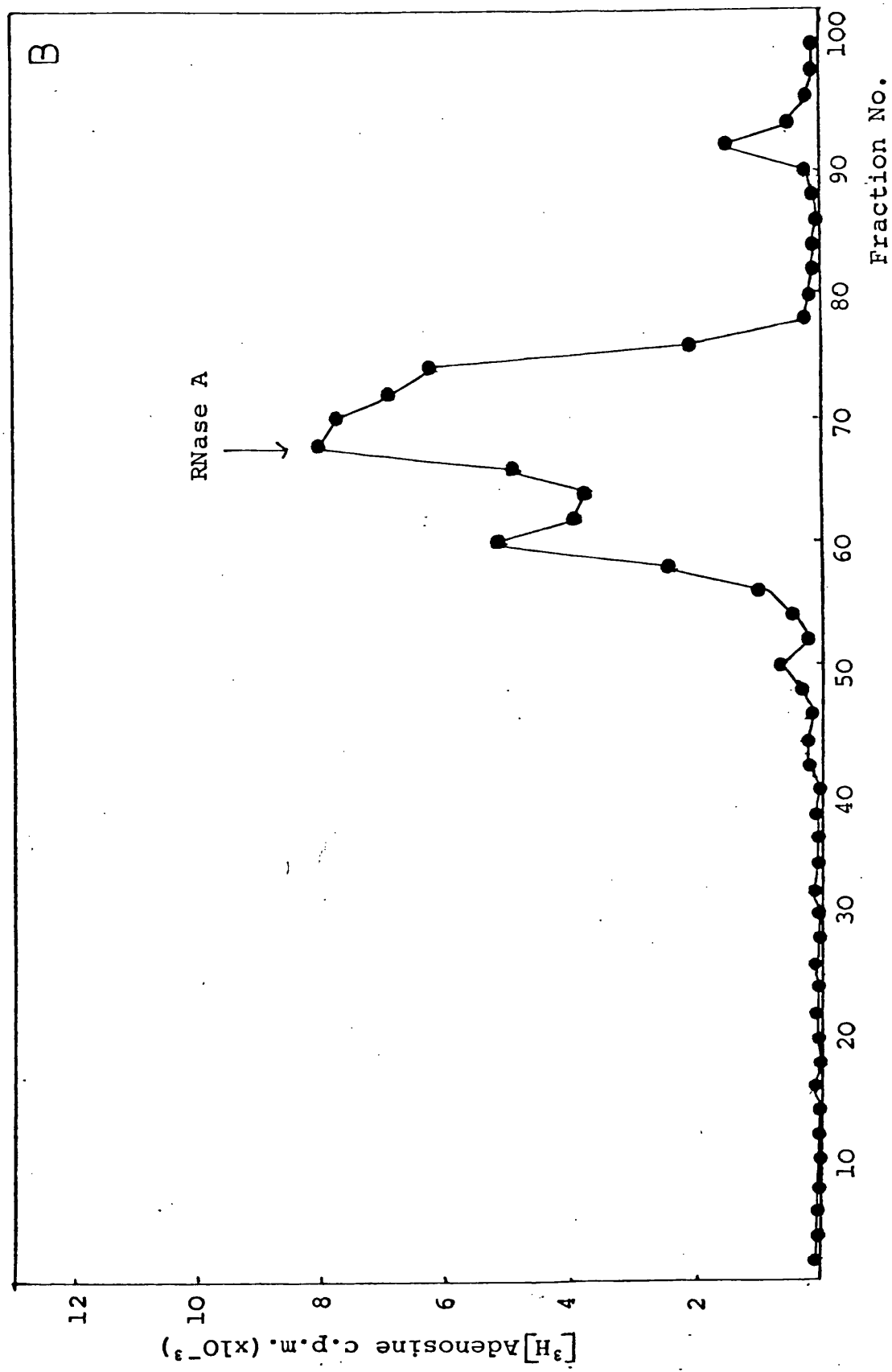


Fig. 3.6.2B



and non-selective.

3.7 DISCUSSION

Caesium chloride density gradient centrifugation in the presence of urea permitted a good separation between RNA and DNA, and putative ADP-ribosylated protein. About 13% of [^3H]adenosine-labelled acid-insoluble material banded at the top of the gradient in association with protein and 87% banded at the bottom of the gradient. The distribution of macromolecular material on the gradient was established by labelling cells with [^3H]thymidine, [^3H]uridine and [^3H]leucine. 96% and 93% of [^3H]thymidine-labelled and [^3H]uridine-labelled acid-insoluble material, respectively, banded at the bottom of the gradient. 96% of [^3H]leucine-labelled acid-insoluble material banded at the top of the gradient. This strongly suggests that the [^3H]adenosine-labelled material at the top of the gradient was covalently associated with protein. To achieve this clear-cut separation it was necessary to treat the material with 10M urea before centrifugation to dissociate any strong ionic bonds between protein and DNA or RNA. The procedure adopted for harvesting the cells in preparation for centrifugation exploited the acid-insolubility of DNA, RNA and protein in TCA and acetone. The method ensured both an immediate inactivation of degradative enzymes and a quantitative recovery of radiolabelled macromolecular species. Only about 5% of acid-insoluble radioactivity was lost throughout the whole procedure. Furthermore, a negligible amount of proteolysis, about 3%, was

detectable under the conditions of centrifugation. This method provided a non-selective and quantitative means of isolation of putative ADP-ribosylated protein which was both simple and reproducible. The relatively few steps involved in the isolation ensured that little loss or error was introduced into the system.

To date no other method has permitted the quantitative and non-selective isolation of ADP-ribosylated proteins. Okayama et al. (1978) and Adamietz et al. (1979) both developed a method for the specific isolation of in vitro ADP-ribosylated proteins using boronate columns which could be adapted for in vivo radiolabelled ADP-ribosylated proteins. However, the procedure did not allow the quantitative recovery of ADP-ribosylated proteins because the pH employed during chromatography was sufficiently high enough to allow a significant loss of ADPR from protein. The pH value of 5 employed throughout the procedure developed here ensured that this ADPR-protein linkage was always kept intact. Ueda et al. (1975) and Adamietz et al. (1978c) attempted an analysis of ADP-ribosylated protein in vivo. Both groups, however, confined their studies to the relatively easily isolated and well characterized nuclear proteins, histones. The conditions necessary for the isolation of some other proteins e.g. alkaline pH, would destroy the linkage between ADPR and protein. The radioimmunoassay developed by Minaga et al. (1978) was limited in that it was only capable of detecting ADP-ribosylated protein of chain length greater than 4. The ADP-ribosylation of proteins

by one or very few ADPR residues may be extensive (Stone et al., 1976; Ferro et al., 1978). Young & Sweeney (1978, 1979) were able to analyse total protein ADP-ribosylation in the unfertilized mouse ovum and one-cell embryo but the relatively easy isolation and detection of these species was dependent to an extent on the lack of DNA and RNA synthesis at the time of isolation. The method was limited in that it could not be easily adapted for isolation of ADP-ribosylated protein in other systems. The method developed here was non-selective with respect to both the proteins being isolated and the chain length of ADPR on these proteins. Furthermore, it was not dependent on any special characteristics of the cells employed and as such could be adopted for other systems. Only in this way can a comprehensive comparison of ADP-ribosylation in vivo be made with that in vitro. The method developed here allows not only a direct comparison of ADP-ribosylation in vivo and in vitro in L1210 cells but also, because it can be adapted for many different systems, it potentially allows a general comparison of ADP-ribosylation to be made under these conditions.

CHAPTER 4

C H A R A C T E R I Z A T I O N O F P R O T E I N

A D P - R I B O S Y L A T I O N I N V I V O

4.1 INTRODUCTION

In the previous section the development of a method which allowed the isolation of putative ADP-ribosylated proteins from DNA and RNA was described. The next step was to characterize the [³H]adenosine-labelled macromolecular species to establish whether the label was indeed incorporated into ADPR bound to protein. Since the isolation method developed was a non-selective one, the characterization of 'total' proteins ADP-ribosylated in vivo was made possible. Furthermore, since the aim of this work was to analyse the ADP-ribosylation of protein in vivo and so compare it with that in vitro, it was felt that the best preliminary approach to the characterization of isolated material would be to employ those methods already well established in vitro. In this way a direct comparison of ADP-ribosylation in the two systems could be made.

The identification of poly(ADPR) has been greatly facilitated by its sensitivity to snake venom phosphodiesterase but insensitivity towards other enzymes capable of digesting nucleic acids (Nishizuka et al., 1967). An additional advantage of snake venom phosphodiesterase digestion of poly(ADPR) is that the unique product PR-AMP is produced which may be separated from 5'AMP by a number of chromatographic systems, including paper chromatography (Nishizuka et al., 1967) and ion-exchange thin-layer (Lehmann et al., 1974) or column (Shima et al., 1969) chromatography. The chain length of the polymer may also be calculated from such an analysis (Nishizuka et al., 1969).

The sensitivity of the ADPR-protein bond to alkali and neutral hydroxylamine (Nishizuka et al., 1969) permits the linkage between ADPR and protein to be confirmed. Three types of linkage have been reported thus far: (i) alkali and hydroxylamine-sensitive (Nishizuka et al., 1969), (ii) alkali-sensitive but hydroxylamine-resistant (Adamietz & Hilz, 1976), and (iii) alkali-resistant (Adamietz et al., 1978). The alkali-resistant bond is a new type of linkage recently found in histone H1 ADP-ribosylated in vivo.

Identification of the number and type of protein species modified by ADPR may give an indication of the role of ADP-ribosylation in nuclear function. Such analyses have been made mainly by using gel electrophoretic (e.g. Rickwood et al., 1977; Adamietz et al., 1979) and ion-exchange column chromatographic (e.g. Ueda et al., 1975) methods.

The relatively few attempts at characterizing the ADP-ribosylation of protein in vivo have been carried out on easily isolated, well characterized proteins. Such studies have demonstrated that ADP-ribosylation in vivo may differ significantly from that observed in vitro. For example, Adamietz et al. (1978c) compared the ADP-ribosylation of histone H1 from HeLa cells in vitro and in vivo and showed that in vivo material is of much shorter average chain length than that in vitro, that few ADPR-protein linkages in vivo are hydroxylamine-sensitive and that a novel, alkali-resistant linkage between ADPR and protein exists. Furthermore, the amount of ADPR bound to histone H1

in vivo was much less than that in vitro. Whether these differences reflect a general trend or are merely characteristic of the species being analysed (particularly with respect to the different conditions it has been exposed to) can only be determined by analysis of other ADP-ribosylated proteins. However, the isolation conditions necessary for some proteins would result in the release of ADPR from protein. Clearly a method is required which allows the characterization of total proteins ADP-ribosylated in vivo. The isolation method developed here allows such an approach to be carried out.

The characterization of total in vivo ADP-ribosylated protein described below was therefore made with respect to three aspects of the molecule, namely: (i) identification of mono(ADPR)/poly(ADPR), (ii) characterization of ADPR-protein linkage, and (iii) analysis of protein species modified, in an attempt to produce a comprehensive analysis of ADP-ribosylation in vivo which may be directly compared with that in vitro.

4.2. ENZYMIC AND CHEMICAL HYDROLYSES OF ADP-RIBOSYLATED PROTEIN

Acid-insoluble, [^3H]adenosine-labelled material from the top of the caesium chloride gradient, previously precipitated and washed as described in Section 2.2.11, was treated with a number of enzymes and chemicals to determine the nature of the labelled species isolated. In preparation for these analyses the final acetone pellet was dissolved in water or buffer. However, under these

conditions a large amount of material remained insoluble. Attempts at hydrolysis under these conditions gave a lower percentage of hydrolysis than expected e.g. only 52% of acid-insoluble material was converted to the acid-soluble form on treatment with snake venom phosphodiesterase. It seemed that the putative ADP-ribosylated protein may have been aggregated and as a result caused a reduced accessibility of the enzyme or chemical to sites of hydrolysis. By dissolving the sample in 5M urea prior to treatment this problem was overcome. Since some enzymes may be inhibited in the presence of urea, the volume of urea was kept low with respect to that of the final digest.

Treatment with snake venom phosphodiesterase, alkaline phosphatase and NaOH was carried out as described in Section 2.2.12. Other hydrolyses were carried out under the following conditions: (i) spleen phosphodiesterase - 0.2 I.U. enzyme, 50mM sodium phosphate - pH 6.5, 10 mM $MgCl_2$, 2h, 37°C, (ii) RNase A - 20 Kunitz units enzyme, 50mM Tris/HCl - pH 7.5, 2h, 37°C, (iii) DNase I - 20 Kunitz units enzyme, 50mM Tris/HCl - pH 7.5, 10mM $MgCl_2$, 2h, 37°C, (iv) Pronase - 0.2mg enzyme, 50mM Tris/HCl - pH 7.5, 2% (w/v) SDS (Hilz et al., 1975), 2h, 37°C, (v) poly(ADPR) glycohydrolase - 40µl enzyme, 100mM Tris/HCl - pH 8.2, 10mM $MgCl_2$, 60mM KCl, 4mM KF, 2mM DTT, 2h, 37°C, (vi) 50mM sodium phosphate - pH 6.5, 30 minutes, room temperature, (vii) hydroxylamine - 0.8M-pH 7.5, 2h, 37°C. Controls for each enzyme digest contained all ingredients of the digest except the enzyme. Control for chemical hydrolyses was 100mM Tris/HCl - pH 7.1. The results of this preliminary

Table 4.2 Enzymic and Chemical Analyses of [³H]Adenosine-Labelled Material

Treatment	c.p.m. Before Treatment	c.p.m. After Treatment	% Radioactivity Digested
1. Control	10,878 ± 199	10,552 ± 185	3
2. Snake Venom Phosphodiesterase	10,025 ± 180	2,907 ± 106	71
3. Snake Venom Phosphodiesterase + Urea	10,558 ± 172	845 ± 147	92
4. Snake Venom Phosphodiesterase + Alkaline Phosphatase	9,992 ± 185	700 ± 52	93
5. Spleen Phosphodiesterase	10,734 ± 202	8,909 ± 160	17
6. Ribonuclease A	10,268 ± 218	8,830 ± 192	14
7. Deoxyribonuclease I	9,887 ± 198	9,689 ± 203	2
8. Pronase	10,126 ± 183	3,240 ± 131	68
9. Glycohydrolase	10,882 ± 220	6,638 ± 158	39
10. Phosphate Buffer, pH 6.5	10,522 ± 201	10,312 ± 196	2
11. Sodium Hydroxide	10,436 ± 193	1,357 ± 97	87
12. Hydroxylamine	10,071 ± 184	7,956 ± 153	21

The TCA precipitated, [³H]adenosine-labelled material from the top of the gradient was dissolved in 0.5ml 5M urea and 25µl samples used for each treatment. Treatment 4 involved alkaline phosphatase digestion of 50µl of the snake venom phosphodiesterase digest. The final volume of each digest was 200µl. Acid-insoluble radioactivity was determined on paper discs as described in Section 2.2.3. Percentage radioactivity digested was calculated with respect to zero time. Radioactivity represented is that of the total digest. Values for treatment 4 have been normalized with respect to the other treatments. Each value is the mean of three samples and the error is represented as the standard error of that mean (S.E.M.).

characterization are shown in Table 4.2.

4.2.1 Enzymic Hydrolysis

Of the enzymes employed only snake venom phosphodiesterase and Pronase rendered the incorporated radioactivity acid-soluble to any significant extent, 92% and 68% of material being digested by these enzymes respectively. The reported stimulation of spleen phosphodiesterase and RNase by urea (Hilz et al., 1975) prompted a comparison of snake venom phosphodiesterase digestion in the presence or absence of urea to be made. Analysis of the decrease in acid-insoluble radioactivity with time (see Fig. 4.2.1) showed that a greater degree of digestion, 92% compared to 71%, was in fact observed in the presence of urea. Consequently, all further digestions by this enzyme were carried out in the presence of 4M urea.

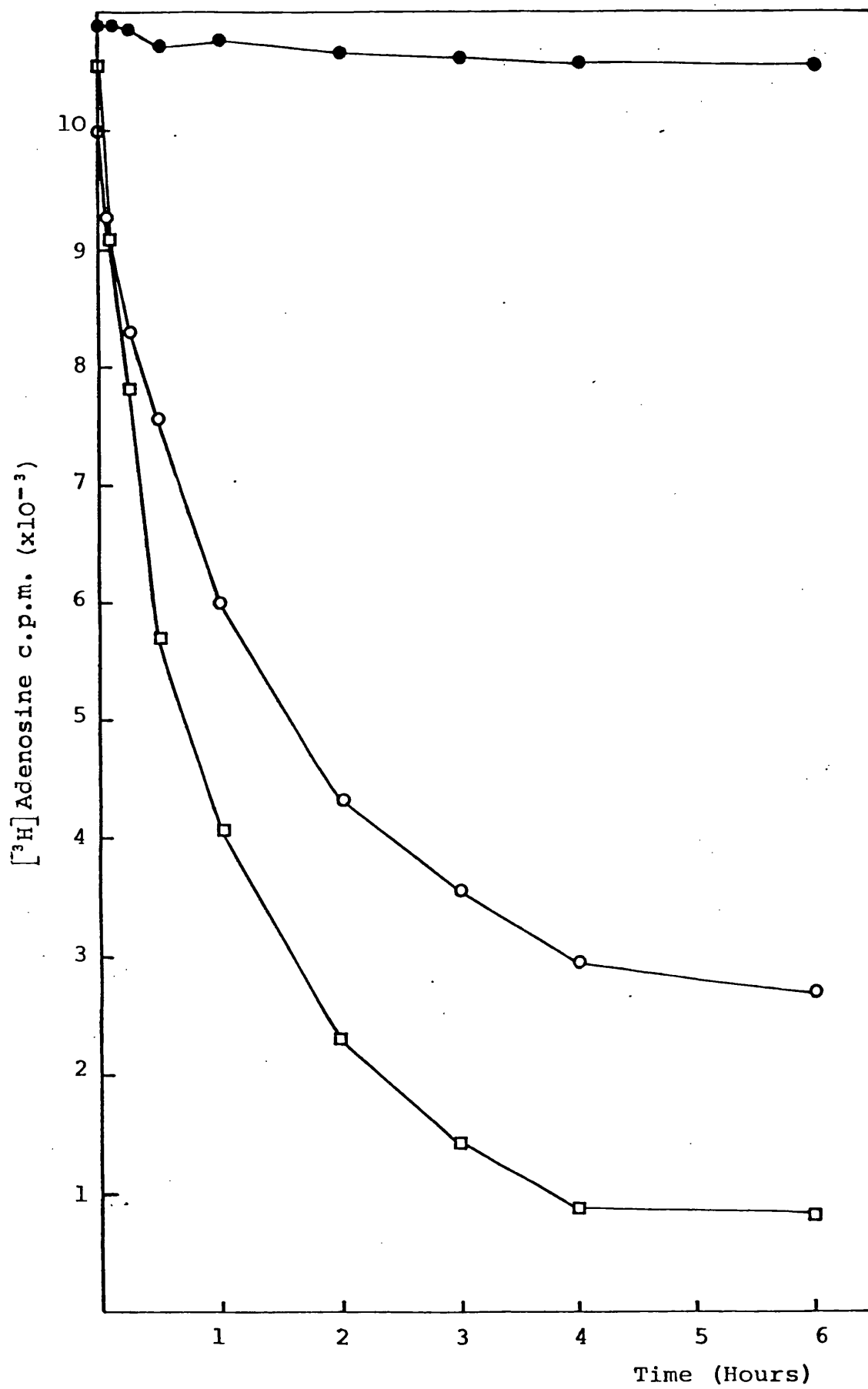
Digestion with spleen phosphodiesterase and RNase A rendered a small amount, 17% and 14%, respectively, of material acid-soluble. None of the labelled material appeared to be susceptible to DNase I. Furthermore, treatment with buffer at pH 6.5, a condition which dissociates an AMP-protein complex detected in HeLa cells by Stone & Kidwell (1980), showed no release of acid-soluble material.

If the labelled material was poly(ADPR) bound to protein then incubation with its physiological degradative enzyme, poly(ADPR) glycohydrolase, should release acid-soluble radioactivity. On treatment with a crude glycohydrolase preparation (prepared by Dr. W.J.D. Whish and

Fig. 4.2.1. Time Course of Digestion of [³H]Adenosine-
Labelled Material with Snake Venom
Phosphodiesterase

At various time points a 20 μ l aliquot of the digest was taken and acid-insoluble radioactivity (on paper discs) determined as described in Section 2.2.3. Radioactivity represented is that of the total digest. —●— control, —○— snake venom phosphodiesterase, —□— snake venom phosphodiesterase and urea.

Fig. 4.2.1



free of both poly(ADPR) synthetase and phosphodiesterase activities) 39% of radioactivity was rendered acid-soluble. The specificity of this enzyme, with respect to the ribose-ribose bond, made it rather unlikely that other [^3H]adenosine-labelled material could have been digested in this way.

4.2.2 Chemical Hydrolysis

Treatment of the [^3H]adenosine-labelled material with NaOH resulted in the majority, but not all, of the radioactivity being converted to the acid-soluble form; about 13% remained acid-insoluble. This acid-insoluble material may represent poly(ADPR) of long chain length and/or alkali-resistant ADP-ribosylated protein. Incubation with neutral hydroxylamine released only 21% of radioactivity in the acid-soluble form, a value much lower than that released by alkali. A high percentage of putative poly(ADPR) or mono(ADPR) appeared to be linked to proteins via a hydroxylamine-resistant bond. Some of this material, however, may have been long chain poly(ADPR) which is intrinsically acid-insoluble. The decrease in acid-insoluble radioactivity with time on incubation with NaOH or hydroxylamine is shown in Fig.

4.2.2.1.

All enzymic and chemical hydrolyses were followed by measuring the increase in acid-soluble radioactivity released with time. The results of this analysis are shown in Fig. 4.2.2.2 and agree well with those obtained from the determination of decrease in acid-insoluble

Fig. 4.2.2.1 Time Course of Hydrolysis of [³H]Adenosine-
Labelled Material with Sodium Hydroxide and
Neutral Hydroxylamine

At various time points a 20 μ l aliquot of the digest was taken and acid-insoluble radioactivity (on paper discs) determined as described in Section 2.2.3. Radioactivity represented is that of the total digest. —●— control, —○— NaOH, —■— NH₂OH.

Fig. 4.2.2.1

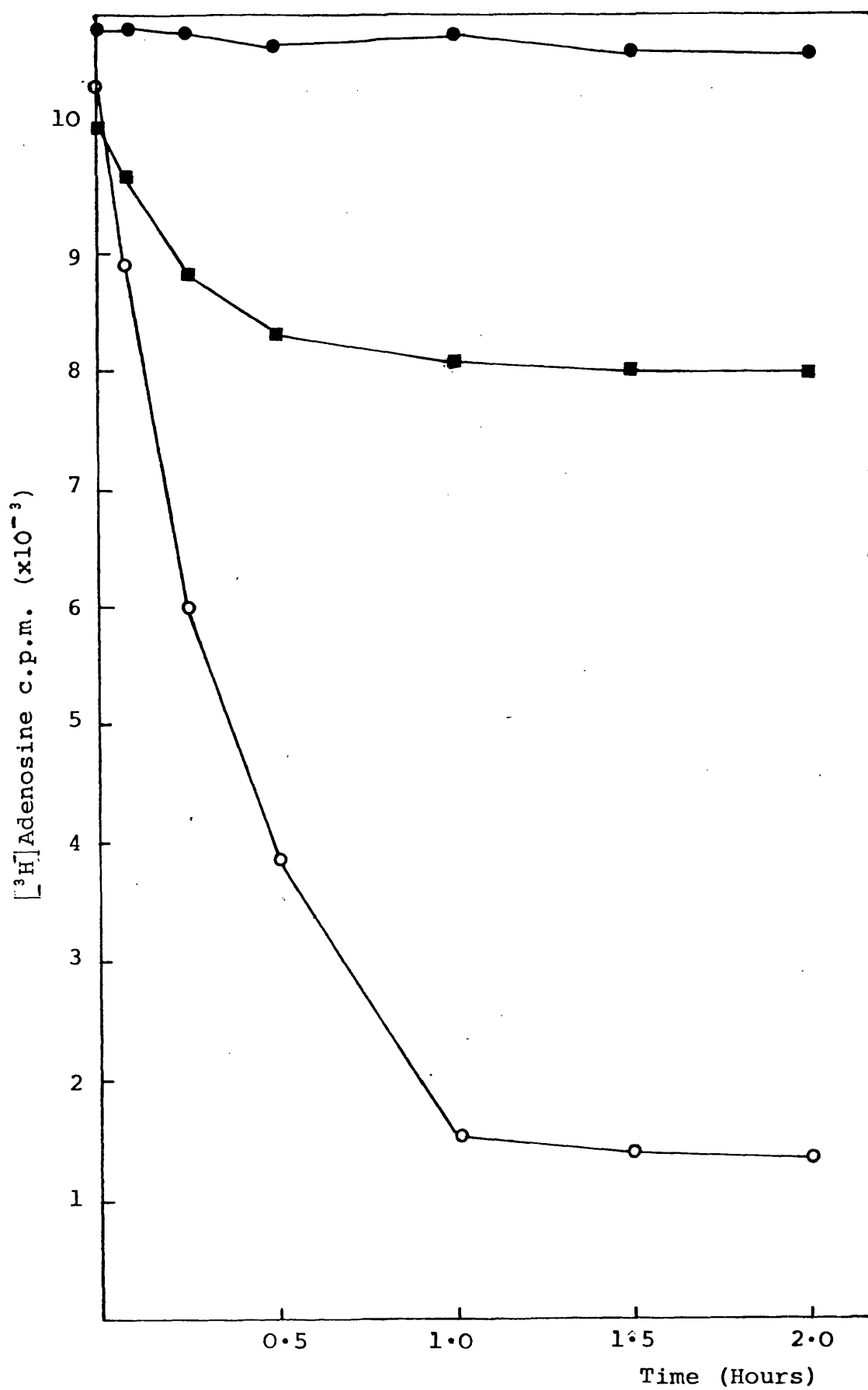
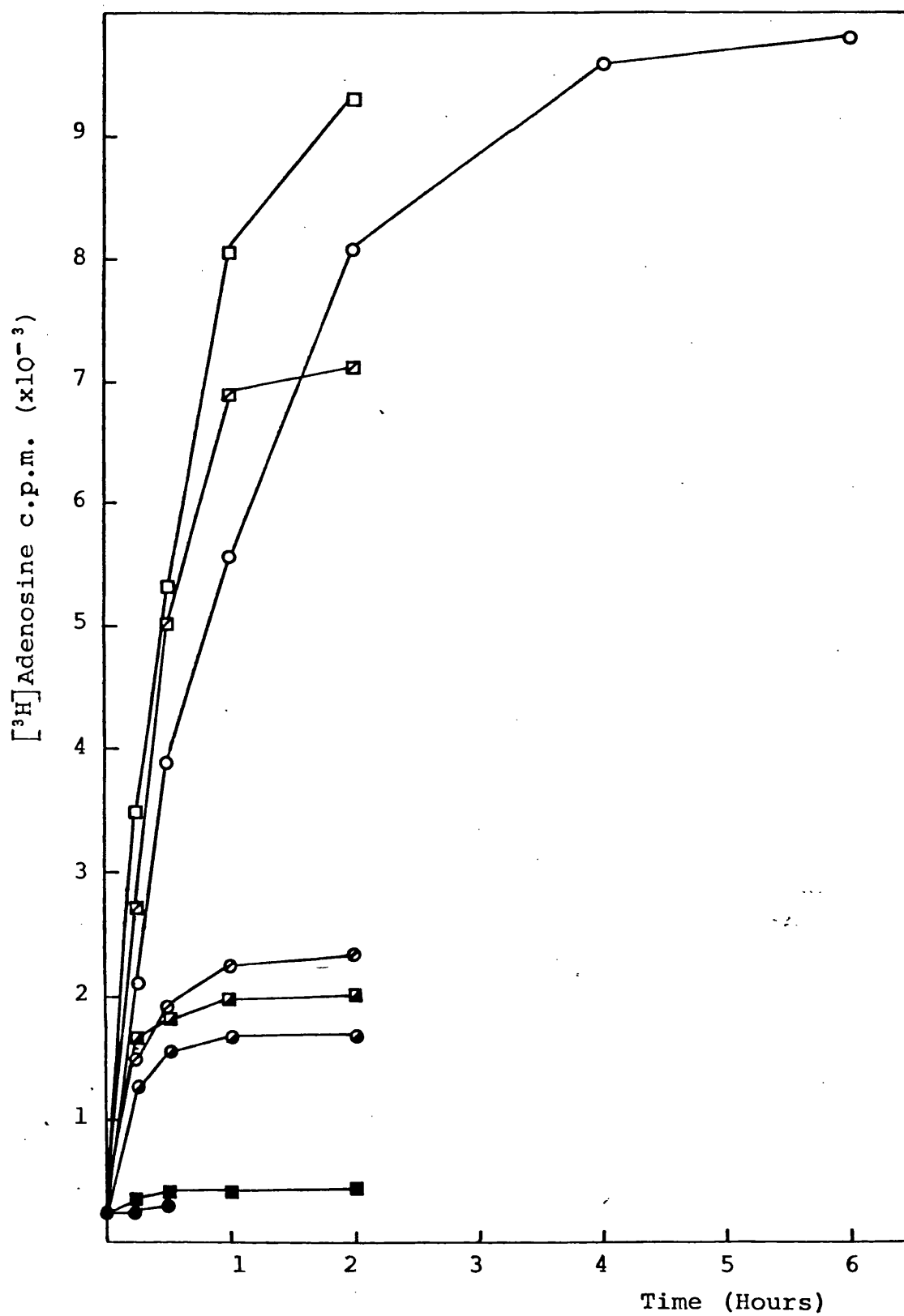


Fig. 4.2.2.2 Increase in Acid-Soluble Radioactivity
During Enzymic and Chemical Hydrolysis

20 μ l aliquots of the digest were centrifuged at various time points, at 10,000g and 4°C, in 66% (v/v) EtOH, 50mM NaAc/HAc - pH5 for 10 minutes and the supernatant analysed for total radioactivity as described in Section 2.2.3. Radioactivity represented is that of the total digest. —○— snake venom phosphodiesterase (and urea), —■— spleen phosphodiesterase, —●— RNase A, —■— DNase I, —□— Pronase, —□— NaOH, —○— NH₂OH, —●— sodium phosphate buffer, pH 6.5.

Fig. 4.2.2.2



radioactivity with time.

4.3 TLC ANALYSES OF THE PRODUCTS OF HYDROLYSIS

The preliminary analyses described above gave a good indication of the nature of the [^3H]adenosine-labelled material. To confirm this chromatographic analyses of the products of hydrolysis were carried out. Thin-layer chromatography on PEI-cellulose was chosen because it is a well established method for the separation of nucleosides, nucleotides and their derivatives (Randerath & Randerath, 1967). It is also quick, simple and reproducible thus permitting a large number of samples to be analysed and compared.

4.3.1 T.l.c. Analysis of Snake Venom Phosphodiesterase Digestion Before and After Treatment with Alkaline Phosphatase - Determination of Chain Length

(a) The putative products of snake venom phosphodiesterase digestion of isolated [^3H]adenosine-labelled material, PR-AMP and 5' AMP, were separated by the HAC/HAC-LiCl system of Randerath & Randerath (1965a) (see Section 2.2.13). Since the sample contained 4M urea it was necessary to desalt in anhydrous methanol (see Section 2.2.13) before the plate was developed. 5'GMP acted as a marker for PR-AMP. A representative t.l.c. of the products of snake venom phosphodiesterase digestion is shown in Fig.1 of the Appendix of this thesis. 14.5% of the radioactivity ran as PR-AMP and 75% as 5'AMP. Calculation of the average chain length

according to the method of Nishizuka et al. (1969) gave a value of 1.19 for this sample.

(b) On treatment with alkaline phosphatase the products of snake venom phosphodiesterase digestion, PR-AMP and 5'AMP, are dephosphorylated to give ribosyl-adenosine and adenosine, respectively. The products of alkaline phosphatase digestion were separated by the double-distilled water system described by Miwa et al. (1979) (see Section 2.2.13). A representative t.l.c. of this separation is shown in Fig.2 of the Appendix. 15% of radioactivity ran as ribosyl-adenosine and 75.5% as adenosine. Since these products were directly derived from PR-AMP and 5'AMP, an average chain length value could also be calculated from the respective values obtained. A value of 1.2 was obtained which agrees well with that obtained on digestion with snake venom phosphodiesterase alone.

4.3.2 T.l.c. Analysis of Base Digest.

(a) Base treatment of ADP-ribosylated protein cleaves the linkage between ADPR and protein to release mono(ADPR) and poly(ADPR). If this treatment is prolonged any mono(ADPR) released may be further converted to 5'AMP, although the conversion is not quantitative (Goebel et al., 1977). T.l.c. analysis of the products of such prolonged treatment should therefore confirm the nature of the acid-soluble material released because of the specificity of the conversion. However, the results of the preliminary

analysis revealed that some RNA may be present at the top of the gradient; a small percentage of material was susceptible to spleen phosphodiesterase and RNase A. On prolonged treatment with base, RNA is cleaved to produce 3'AMP. Thus, to determine the amount of radioactive AMP derived from mono(ADPR) it was necessary to analyse the products of prolonged base digestion in a chromatographic system capable of separating 3'AMP and 5'AMP. In this way the sensitivity of the ADPR-protein linkage to NaOH could be confirmed and, in addition, the presence of any RNA could be established.

Initially two systems were employed: (i) the HAc/LiCl system of Randerath & Randerath (1965b), and (ii) the LiCl/EtOH/(NH₄)₂BO₃ system of Schwartz & Drach (1975) (see Section 2.2.13). The latter of these two systems exploits the cis-diol on ribose in 5'AMP to form a borate complex. The mobility of this complex on PEI-cellulose is retarded compared to that of 3'AMP (see Section 2.2.13 and Fig. 2.2.13.3). Representative t.l.c.s. of these runs are shown in Figs. 3 and 4 of the Appendix. In the borate system 52% of radioactivity ran as 5'AMP, 18% ran as 3'AMP and 24% remained at or near the origin. In the HAc/LiCl system 57% of radioactivity ran as 5'AMP, 14% ran as 3'AMP and 21.5% remained at the origin. The results of the two different analyses agree very well with each other. Radioactivity on the origin represented free oligo(ADPR) and poly(ADPR) and/or alkali-resistant ADP-ribosylated protein.

Since not all ADPR is converted to 5'AMP on treatment

with base overnight, an additional chromatographic system was employed which would allow not only a good separation of 5'AMP and 3'AMP, but also a separation of ADPR from 5'AMP (in the previous systems employed the position of these two compounds overlapped). The $\text{H}_3\text{BO}_3/\text{Na}_2\text{B}_4\text{O}_7$ system described by Young and Sweeney (1978) was chosen and a representative t.l.c. of such a run is shown in Fig.5 of the Appendix. In this way the percentage conversion of ADPR to 5'AMP could be determined. 46% of radioactivity ran as 5'AMP, 9% ran as ADPR, 17% ran as 3'AMP and 22.5% remained at or near the origin. From these values the percentage conversion of ADPR to 5'AMP was estimated to be about 84%. The average of 5 samples gave a value of about 81.5% conversion.

From the results of these t.l.c. analyses base digestion was estimated to produce, on average, 53.5% 5'AMP, 16.3% 3'AMP and 22.7% oligo(ADPR), poly(ADPR) or alkali-resistant ADP-ribosylated protein (radioactivity remaining on origin).

(b) The results obtained above suggested the presence of a small amount of RNA. To test this possibility further 5'AMP and 3'AMP were enzymically converted to adenosine by incubation with 5' nucleotidase and 3' nucleotidase, respectively. A 50 μ l sample, previously hydrolysed by base, was neutralized with 1M HAC and then incubated with either one of the following: (i) 0.2 I.U. 5' nucleotidase, 50mM Tris/HCl-pH 8.9, 10mM MgCl_2 , (ii) 0.2 I.U. 3' nucleotidase, 50mM NaAc/HAC - pH 5.0, 1mM ZnCl_2 , 1mM DTT.

The final volume of each sample was 200 μ l. Digestion was for 2h at 37°C.

Radioactive adenosine derived from these digests was then analysed by t.l.c. using the HAc/HAc-LiCl system of Randerath & Randerath (1965a). Representative t.l.cs. after enzymic conversion are shown in Figs. 6 and 7 of the Appendix. On treatment with 5'nucleotidase 59% of radioactivity ran as adenosine whereas on treatment with 3'nucleotidase only 20% ran as adenosine. These results extend and confirm those described in part (a) above.

A summary of t.l.c. analyses of base digestion is given in Table 4.3.2. The results obtained by these different methods agree well with respect to both the polymer/monomer ratio and the amount of RNA present.

4.3.3 T.l.c. Analysis of Hydroxylamine Digest

Hydroxylamine treatment of ADP-ribosylated protein cleaves some of the linkages between ADPR and protein to release mono(ADPR) or oligo(ADPR). Preliminary analysis, on the basis of release of acid-soluble radioactivity, showed 21% to be sensitive to hydroxylamine. T.l.c. analysis was carried out to confirm that ADPR was released. A representative t.l.c. of the product of hydrolysis is shown in Fig.8 of the Appendix. 17% of radioactivity on the plate ran as ADPR, thus confirming the hydroxylamine-sensitivity of the ADPR-protein linkage in vivo. The remaining radioactivity did not run but stayed at or near the origin. No ADPR was detected on a control run using 100mM Tris/HCl - pH 7.1. The value of hydroxylamine-

Table 4.3.2 Comparison of TLC Analysis on
PEI-Cellulose of Base Digest

Method of Analysis	% Radioactivity			
	Origin	5'AMP	3'AMP	Adenosine
I	24	52	18	-
II	21.5	57	14	-
III	22.5	51.5	17	-
IV	22.5	54.5	-	20
V	21	-	17	59

The methods of analysis are described in the legends of Figs. 3 - 7 of the Appendix.

sensitive ADPR-protein linkages is lower than that detected by the acid-insolubility test because any dimers, trimers etc., of ADPR which were acid-soluble in the preliminary analysis would remain at or near the origin on t.l.c. analysis.

4.3.4 T.l.c. Analysis of Crude Glycohydrolase Digest

Thin-layer chromatographic analyses of the snake venom phosphodiesterase digest (in the absence or presence of alkaline phosphatase) clearly suggested the presence of ADP-ribosylated protein at the top of the gradient. However, to prove unambiguously the nature of this material it was felt that thin-layer chromatographic analysis of the product of digestion by the physiologically specific degradative enzyme of poly(ADPR), poly(ADPR) glycohydrolase, was necessary. T.l.c. analysis of digestion by a crude preparation of the glycohydrolase is shown in Fig. 9 of the Appendix. 51% of radioactivity on the plate remained at the origin, 17% ran as ADPR and 27% ran as AMP. The presence of radioactive ADPR confirms that the material bound to protein is indeed poly(ADPR). Since poly(ADPR) glycohydrolase does not cleave the last ADPR residue attached to protein, the radioactive material at the origin represents mono(ADP-ribosyl)ated protein. The presence of AMP on the plate is probably due to contaminating enzymes in the preparation.

4.3.5 T.l.c. Analysis of Deoxyribonuclease/Snake Venom Phosphodiesterase/Alkaline Phosphatase Digest

Preliminary analysis of [^3H]adenosine-labelled acid-insoluble material by digestion with DNase I showed no release of acid-soluble material. However, the predominant product of DNase digestion is in fact oligonucleotides. Consequently, such species may not have been detected in an assay for the determination of release of acid-soluble radioactivity. This prompted a more exhaustive analysis by enzymic digestion, using a combination of DNase I, snake venom phosphodiesterase and alkaline phosphatase, in an attempt to prove unambiguously the absence of DNA on top of the gradient. 25 μl of TCA precipitated, [^3H]adenosine-labelled material previously dissolved in 0.5ml 5M urea, were digested overnight at 37°C with 0.2 I.U. snake venom phosphodiesterase, 0.2 I.U. alkaline phosphatase, and 20 Kunitz units DNase I. The incubation mixture also contained 50mM Tris/HCl - pH 7.5, 10mM MgCl_2 in a final volume of 200 μl .

If DNA were present then the final product of this digestion would be deoxyadenosine. Since previous t.l.c. analyses have shown the presence of mono(ADPR)-protein (and a small amount of RNA), then adenosine will also be produced on digestion by these enzymes. Thus, the borate t.l.c. system of Schwartz & Drach (1975) was employed so that any deoxyadenosine produced from DNA could be separated from adenosine produced from the other [^3H]-adenosine-labelled species present. T.l.c. analysis of the products of this digestion is shown in Fig.10 of the

Appendix. 66% of radioactivity on the plate ran as adenosine and 15%, presumably ribosyl-adenosine from poly(ADPR), ran close behind it. Since deoxyadenosine cannot form a borate complex, it would be expected to run above adenosine in the t.l.c. system employed. No radioactivity above basal level was detected in this region and so it was concluded that no DNA was present at the top of the gradient.

4.4 CAESIUM CHLORIDE DENSITY GRADIENT ANALYSIS OF BASE DIGEST

From the results of the preliminary analysis only 87% of [^3H]adenosine-labelled material appeared to be converted to the acid-soluble form on treatment with base. This remaining acid-insoluble material may be a result of the intrinsic acid-insolubility of free poly(ADPR) or, alternatively, may represent ADP-ribosylated protein resistant to alkali. Since a chain length of about 5 ADPR units is necessary before acid-insolubility is exhibited, the possibility that the remaining material could be totally attributed to free poly(ADPR) was rather unlikely, particularly when the estimated average chain length value was taken into account. If all the acid-insoluble material represented alkali-resistant ADP-ribosylated protein, then (correcting for RNA) a maximum value of 15% was calculated. However, the material was likely to be a combination of these two alternatives. Since alkali-resistant ADP-ribosylated protein could not be distinguished from free poly(ADPR) or oligo(ADPR) on t.l.c., this prompted a re-analysis of [^3H]adenosine-labelled

material, after base hydrolysis, by caesium chloride density gradient centrifugation. In this way these species could be separated on the basis of their different buoyant densities. The results of this analysis are shown in Fig. 4.4.

The presence of a peak of radioactivity at the position of protein-bound ADPR, accounting for about 55% of acid-insoluble radioactivity on the gradient, suggested that an alkali-resistant linkage between ADPR and protein may exist in vivo and that about 7% of ADPR residues are bound to protein by such a linkage. The presence of only a small peak of radioactivity at the predicted position of free poly(ADPR) on the gradient may be due to the diffusion of ADPR molecules of relatively small chain length.

4.5 AMINOETHYL CELLULOSE COLUMN CHROMATOGRAPHY OF BASE DIGEST

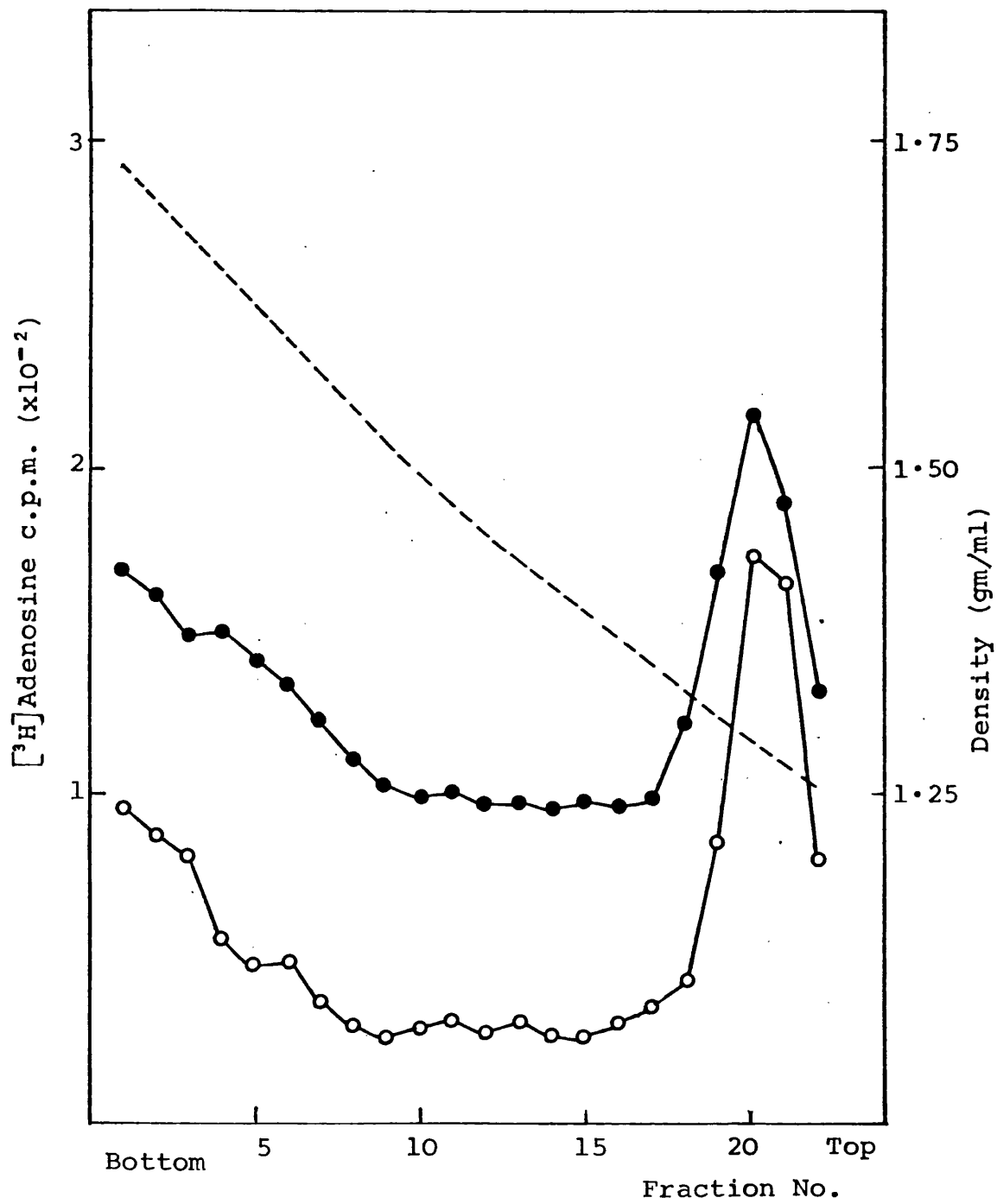
Treatment of ADP-ribosylated protein with base overnight cleaves mono(ADPR) and poly(ADPR) from protein and further cleaves mono(ADPR) to 5'AMP. Because of the differing charge properties of 5'AMP and poly(ADPR), the two products may be potentially separated. This prompted a separation by ion-exchange chromatography using the anion-exchanger aminoethyl cellulose.

Because of its volatility and therefore easy removal ethylamine was employed as the base rather than NaOH. The degree of hydrolysis of the ADPR-protein bond by ethylamine was compared with that produced by NaOH by

Fig. 4.4 Caesium Chloride Density Gradient Analysis
 of Base Digest

The base digest was precipitated with TCA to a final concentration of 20% (w/v) and then washed as described in Section 2.2.9. The final pellet was dissolved in CsCl/urea and centrifuged as described in Section 2.2.10. The distribution of total and acid-insoluble radioactivity (on paper discs) was determined as described in Section 2.2.3. —●— total radioactivity, —○— acid-insoluble radioactivity.

Fig.4.4



determining the decrease in acid-insolubility with time. Fig. 4.5.1 shows that hydrolysis is essentially the same as that with NaOH.

Aminoethyl cellulose chromatography was carried out as described in Section 2.2.17. A representative profile of the radioactivity eluted during chromatography is shown in Fig. 4.5.2. About 71% of the radioactivity eluted in the 6M acetic acid fraction and 29% in the 1M ethylamine fraction.

To confirm that aminoethyl cellulose chromatography did allow the separation of 5'AMP and poly(ADPR) the acetic acid and ethylamine fractions were analysed by PEI-cellulose chromatography. Chromatography of the acetic acid fraction, using the borate system of Schwartz & Drach (1975), is shown in Fig.11 of the Appendix. 66% of the radioactivity ran as 5'AMP, 22% as 3'AMP and 12% as ADPR. No radioactivity remained at the origin, demonstrating that no poly(ADPR) was eluted in 6M acetic acid. Chromatography of the 1M ethylamine fraction is shown in Fig. 12 of the Appendix. All the radioactivity remained at or near the origin of the plate; no radioactivity ran as ADPR or AMP. This demonstrated that only poly(ADPR) eluted in 1M ethylamine. Aminoethyl cellulose chromatography therefore permitted the separation of ADPR and its derivatives from poly(ADPR). Since the procedure allowed full recovery of radioactivity and also since no radioactivity was present on the origin of the t.l.c. of the 6M acetic acid fraction, any alkali-resistant ADP-ribosylated protein appeared to be eluted with 1M ethylamine.

Fig. 4.5.1 Comparison of Time Course of Hydrolysis
of [³H]Adenosine-Labelled Material with
Sodium Hydroxide and Ethylamine

At various time points a 20 μ l aliquot was taken and acid-insoluble radioactivity determined (on paper discs) as described in Section 2.2.3. Radioactivity represented is that of the total digest. —●— control, —○— NaOH, —□— ethylamine.

Fig. 4.5.1

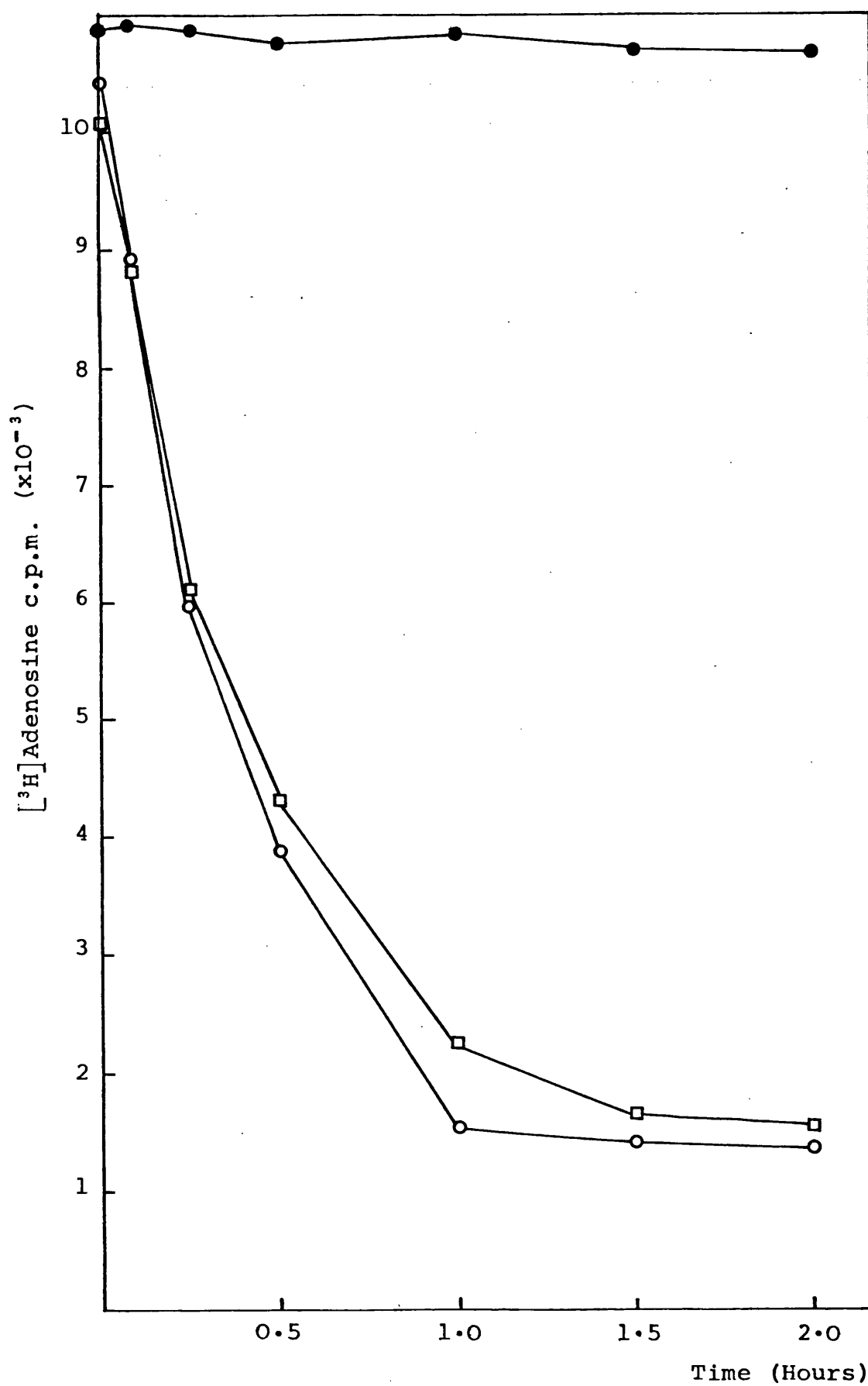


Fig. 4.5.2 Aminoethyl Cellulose Column Chromatography
of Base Digest

The lyophilized ethylamine digest was dissolved in 100 μ l H₂O and applied to an aminoethyl cellulose column (2cm x 0.785 cm²) which had been equilibrated in 6M HAc. Elution was carried out by stepwise application of 6M HAc, H₂O and ethylamine. 1ml fractions were collected and 100 μ l counted for radioactivity as described in Section 2.2.3. The radioactivity represented is that of the total material applied.

Fig.4.5.2

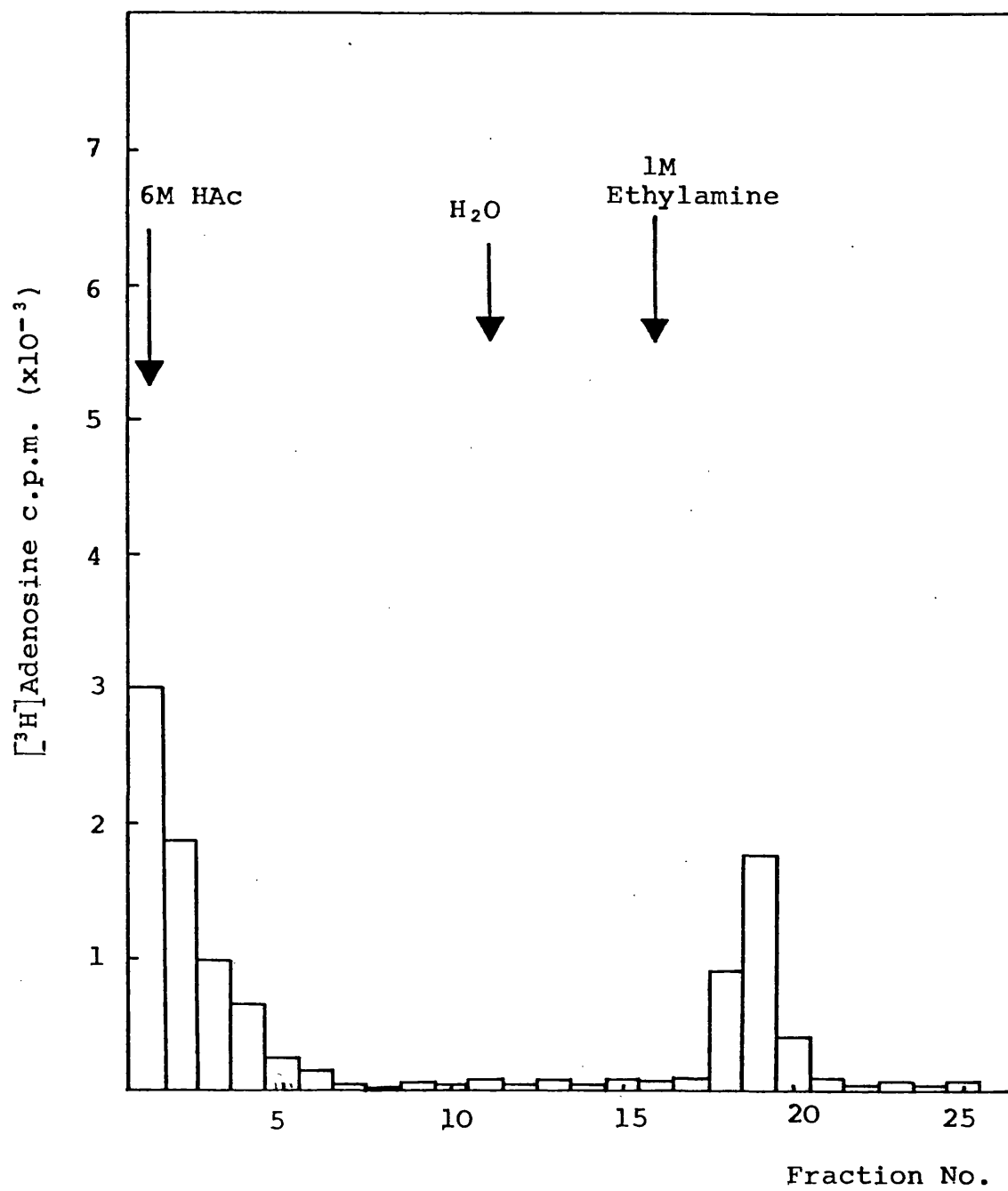


Table 4.5 Chain Length Analyses of ADP-Ribosylated Protein

Treatment	TLC System	Average Chain Length	
		Found	Corrected
S.V.P.D.E. *	1M HAc/0.9M HAc-0.3M LiCl	1.2	1.3
S.V.P.D.E. * + A.P. †	Double- Distilled H ₂ O	1.21	1.3
Base + Aminoethyl Cellulose Chromatography	-	1.18	1.22
Base + Aminoethyl Cellulose Chromatography Then S.V.P.D.E.*	1M HAc/0.9M HAc-0.3M LiCl	2.92	3.3

* Snake Venom Phosphodiesterase

† Alkaline Phosphatase

With the separation of mono(ADPR) and poly(ADPR) by aminoethyl cellulose chromatography, the determination of the average chain length of poly(ADPR) alone was made possible. T.l.c. analysis of a snake venom phosphodiesterase digest of the lyophilized ethylamine fraction is shown in Fig.13 of the Appendix. An average chain length value of 2.92 was obtained. An estimated value, obtained by correcting for 3'AMP and 5'AMP, gave a value of 3.3. The difference in these values may be due, to some extent, to the presence of some alkali-resistant mono(ADP-riboyl)ated protein in the ethylamine fraction.

With the calculated average chain length of poly(ADPR) of 2.92 and the ratio of mono(ADPR)/poly(ADPR) of 2.5 obtained by aminoethyl cellulose chromatography, it was possible to estimate the overall average chain length of ADP-ribosylated protein. This gave a value of 1.18 and, on correcting for 3'AMP, a value of 1.22. These figures agree well with those obtained by previous analyses. A summary of the chain length values of in vivo ADP-ribosylated protein from mouse L1210 cells obtained during this study is given in Table 4.5.

4.6 DISTRIBUTION OF [³H]ADENOSINE INCORPORATED INTO ACID-INSOLUBLE MATERIAL IN L1210 CELLS.

The characterization of [³H]adenosine-labelled material described above allowed a reliable analysis of the distribution of radioactivity incorporated into acid-insoluble material to be made. The results of this analysis are shown in Table 4.6. 0.97% of label incorporated was

Table 4.6 Distribution of [³H]Adenosine Incorporated
Into Acid-Insoluble Material in L1210 Cells

	c.p.m. Incorporated	% Incorporated
Total	$2.72 \times 10^7 \pm 1.50 \times 10^5$	100
Acid-Insoluble	$2.33 \times 10^6 \pm 5.2 \times 10^3$	8.6
Acid-Insoluble on Top of Gradient	$3.09 \times 10^5 \pm 1.13 \times 10^3$	1.14
5'AMP	$2.03 \times 10^5 \pm 7.45 \times 10^2$	0.75
PR-AMP	$5.97 \times 10^4 \pm 2.01 \times 10^2$	0.22

Each value is the mean of 6 samples and the error is represented as the standard error of that mean (S.E.M.).

as ADP-ribosylated protein. 77% of this was in the form of ADPR directly attached to protein (derived from mono (ADPR)-protein or poly(ADPR)-protein) and 22.5% was as extensions of this modification in the form of ADPR of chain length ≥ 2 . Expressed as a percentage relative to acid-insoluble radioactivity this gave values of 8.7% and 2.5%. Thus, 7.83% of acid-insoluble radioactivity was as mono(ADP-ribosyl)ated protein and 3.35% was as poly(ADP-ribosyl)ated protein, giving a total of 11.18% of acid-insoluble radioactivity as ADP-ribosylated protein.

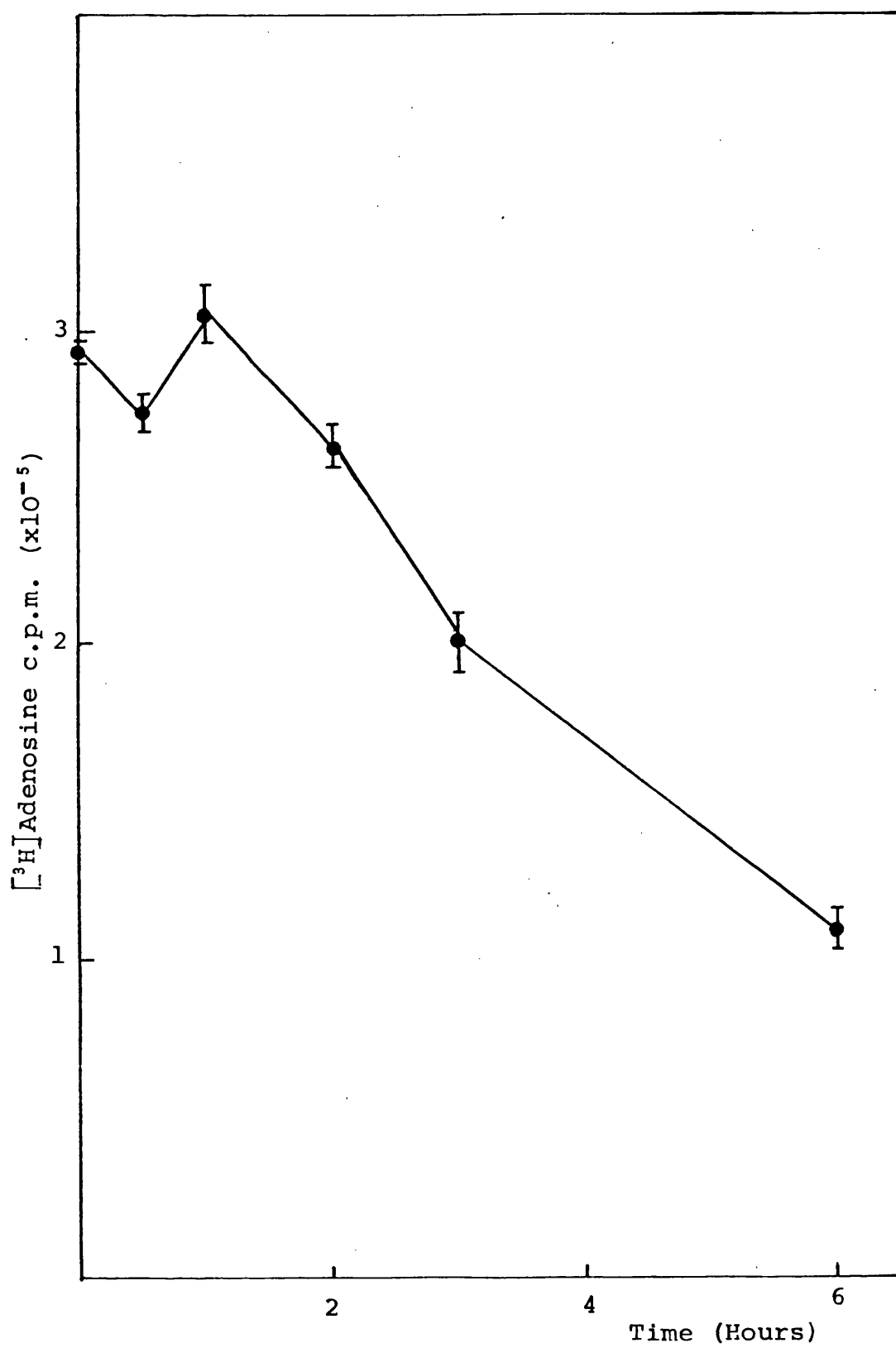
4.7 TURNOVER OF ADP-RIBOSYLATED PROTEIN IN L1210 CELLS

The characterization described above has shown unambiguously that the majority of [^3H]adenosine-labelled material on top of the gradient was ADP-ribosylated protein. Consequently, it was possible to obtain an approximate estimation of the turnover of label on ADP-ribosylated protein by a pulse-chase experiment. Cells labelled with [^3H]adenosine as normal were washed and the label chased with adenosine for up to 6 hours. The decrease in radioactivity with time on top of a caesium chloride density gradient was then followed at various points during this period. The results of this experiment are shown in Fig. 4.7. A surprising finding was the increase in radioactivity observed at one hour after addition of adenosine. This phenomenon was observed each time the experiment was performed and may be attributed to some change in precursor pool levels.

Fig. 4.7 Turnover of ADP-Ribosylated Protein in
Mouse L1210 Cells

Cells were labelled with 40 μ Ci/ml [3 H]adenosine for 18h then centrifuged at 300g and 4°C for 10 minutes and resuspended in fresh medium containing 5 μ M adenosine. Cells were harvested at various time points for up to 6 hours and centrifuged in CsCl/urea as described in Section 2.2.10. Acid-insoluble radioactivity (on paper discs) was determined in Section 2.2.3. Values represented are the mean of three samples and the error is the standard error of that mean. (S.E.M.).

Fig. 4.7



The estimated half-life of ADP-ribosylated protein by this method was about 4.5 hours.

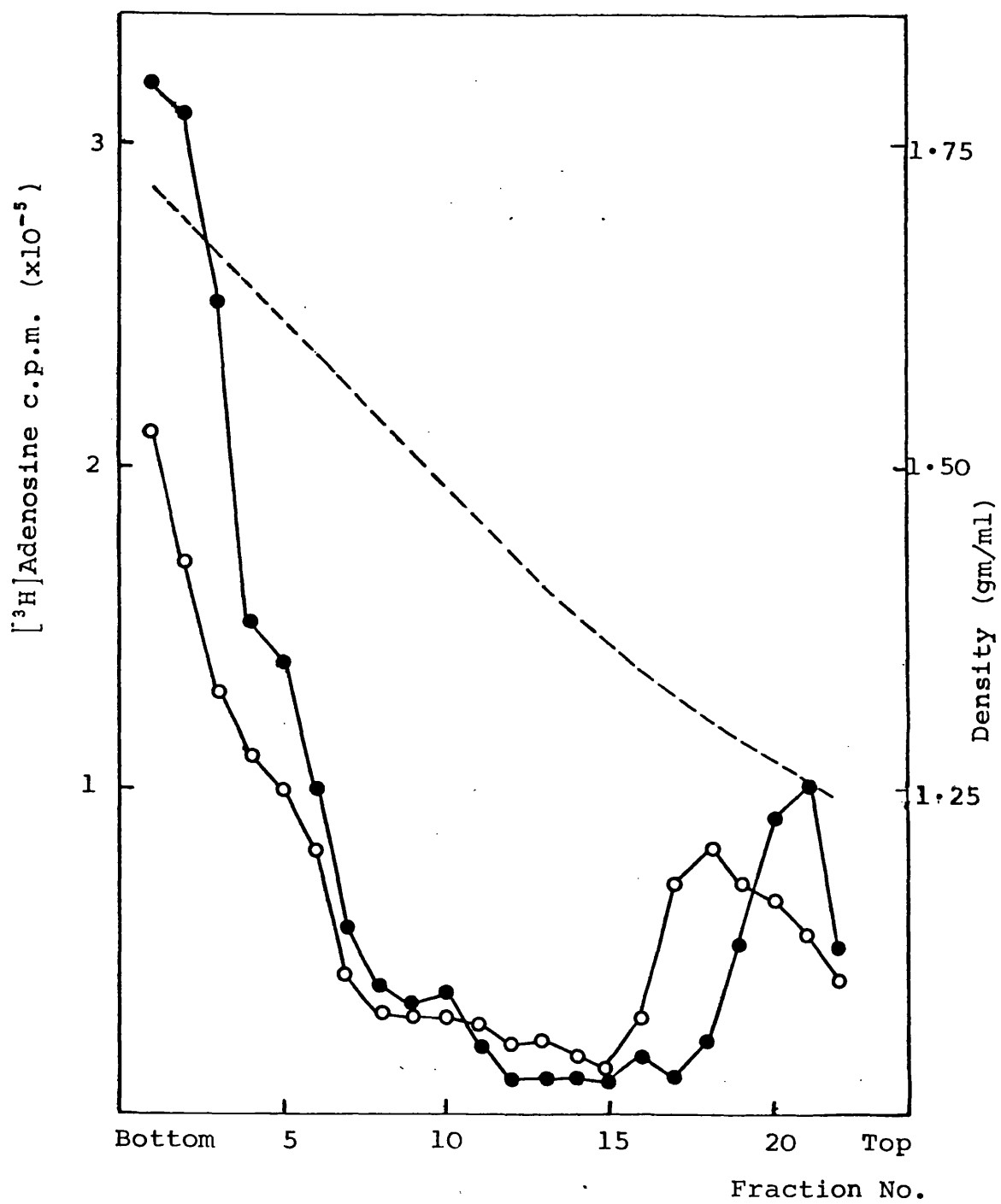
4.8 ADP-RIBOSYLATION OF PROTEINS IN L1210 CELLS FROM STATIONARY PHASE OF GROWTH

The isolation and characterization of proteins ADP-ribosylated in vivo during log phase of L1210 cells showed that the majority of proteins were ADP-ribosylated by a single ADPR residue; only about 11% of acceptor sites were occupied by poly(ADPR). Since the activity of poly(ADPR) synthetase has been shown to increase during log phase (Stone & Shall, 1975) with a maximum value being reached in early stationary phase in both isolated nuclei (Stone & Shall, 1973) and permeabilized cells (Berger et al, 1978b), it was felt that a comparison of ADP-ribosylation during stationary phase with that obtained during log phase would prove invaluable both with respect to the analysis of ADP-ribosylation in vivo and the reproducibility of the isolation method developed. Cells were labelled and harvested as described for those obtained from log phase. The distribution of radioactivity obtained on caesium chloride density gradient centrifugation of [³H]adenosine-labelled stationary phase cells is shown in Fig. 4.8. The pattern of labelling was similar to, but not identical with, that of log phase cells. In log phase cells approximately 13% of acid-insoluble radioactivity banded at the top of the gradient with a peak at a density of about 1.27. In stationary phase cells approximately 23% of acid-insoluble

Fig. 4.8 Comparison of Caesium Chloride Density Gradient
Analysis of Ll210 Cells Labelled with
[³H]Adenosine During Logarithmic Phase and
Stationary Phase

Cells were centrifuged as described in Section 2.2.10.
The distribution of radioactivity was determined (on paper
discs) as described in Section 2.2.3. —●— log phase,
—○— stationary phase, --- density.

Fig. 4.8



radioactivity banded at the top of the gradient with a peak at a density of about 1.32. Furthermore, the distribution of radioactivity within these fractions was broader with stationary phase cells compared with that of log phase cells. In the former case the band ranged from a density of 1.36 to 1.24, whereas in the latter case it ranged from a density of 1.31 to 1.24. Since the density of ADP-ribosylated protein may be affected by the extent to which the protein is modified, this suggests that the ADP-ribosylation of proteins in stationary phase results in chains of longer and more variable length.

4.9 DETERMINATION OF [^{14}C]NICOTINAMIDE-LABELLED ACID-INSOLUBLE MATERIAL

It was realized that some of the [^3H]adenosine-labelled material at the top of the gradient may have been present as protein associated with adenosine and nicotinamide, such as NAD^+ (as coenzyme) tightly bound to the active site of an enzyme. Therefore, to investigate the possibility that some of the results obtained during characterization of gradient material may have been attributable to such species, the percentage incorporation of [^{14}C]nicotinamide into acid-insoluble material was determined. 5ml of L1210 cells were labelled for 18h with 20 μCi [^{14}C]nicotinamide. The medium was aspirated off, TCA added to 20% (w/v) and the sample then left on ice for 2h. The mixture was then filtered and washed through a GF/C disc as described in Section 2.2.2 and the disc counted for acid-insoluble radioactivity. Similarly,

a blank of 20 μ Ci [14 C]nicotinamide alone was filtered and counted. An average of about 2,000 c.p.m. (corrected for blank) was obtained from three samples. Thus, about 0.065% of label added was incorporated into acid-insoluble material. This means that a maximum of about 3.4% (accounting for differing counting efficiencies of 3 H and 14 C) of material on the gradient may be nicotinamide-associated.

4.10 SDS GEL ELECTROPHORESIS

Analysis of the number and type of ADP-ribosylated proteins isolated by CsCl density gradient centrifugation was made by fractionation on SDS gels. The method allows an estimation of the molecular weight of the proteins under conditions which minimize molecular interaction. The method was a modification of that of Weber & Osborn (1969) (see Section 2.2.18). To ensure both maximum resolution of protein bands and stability of the protein-ADPR linkage during electrophoresis, it was necessary to make the following modifications: (i) the system was buffered at pH 6, (ii) a discontinuous buffer system was employed, (iii) the sample was first dissolved in 10M urea for 30 minutes at room temperature and then for a further 2.5h after addition of SDS, phosphate buffer, bromophenol blue, glycerol and β -mercaptoethanol, (iv) gels contained urea in addition to SDS. The sample was left at room temperature in β -mercaptoethanol rather than heated or boiled since the ADPR-protein bond is heat-labile. The long period of incubation in urea and SDS

was necessary to obtain sharply defined protein bands on the gel and may have been the result of the observed insolubility of ADP-ribosylated proteins.

20 μ l samples (about 20 μ g protein, a value equivalent to one fifth of the protein from the top of the gradient) were applied to the top of the gel and electrophoresis carried out until the bromophenol blue marker reached the bottom of the gel. A representative profile of SDS gel electrophoresis of proteins obtained from the top of the gradient is shown in Fig. 4.10. Essentially full recovery of radioactivity was achieved. Eighteen protein bands were observed with molecular weights ranging from about 100,000 to about 2,000. Standard molecular weight markers of bovine serum albumin, ovalbumin, trypsinogen and RNase A ran with R_f values of 0.075, 0.13, 0.24 and 0.33, respectively. R_f values for protein samples are shown in Table 4.10. Radioactivity coincided with a number of these bands. In addition, some peaks of radioactivity did not appear to be coincident with detectable protein bands. It may be that protein present at these positions was below the limit of detection by binding to Coomassie blue. The degree of radioactivity associated with each band varied and did not coincide with the quantity of that particular protein (as determined by degree of staining and relative absorbance). This suggested that the chain length of poly(ADPR) attached to the proteins may have varied. Alternatively, it may be that a greater number of protein molecules of one species were modified compared to that of another. Thus, a number of specific protein

Fig. 4.10 SDS Gel Electrophoresis of Proteins
ADP-Ribosylated In Vivo from Mouse
L1210 Cells

Electrophoresis and determination of the distribution of radioactivity and protein were carried out as described in Section 2.2.18. The anode is on the left of the figure. — Absorbance, -●-radioactivity, -○- standard curve. A - bovine serum albumin (mol.wt. 66,000), B - ovalbumin (mol.wt. 45,000), C - trypsinogen (mol.wt. 24,000), D - Ribonuclease A (mol.wt. 13,700), BPB - bromophenol blue.

Fig 4.10

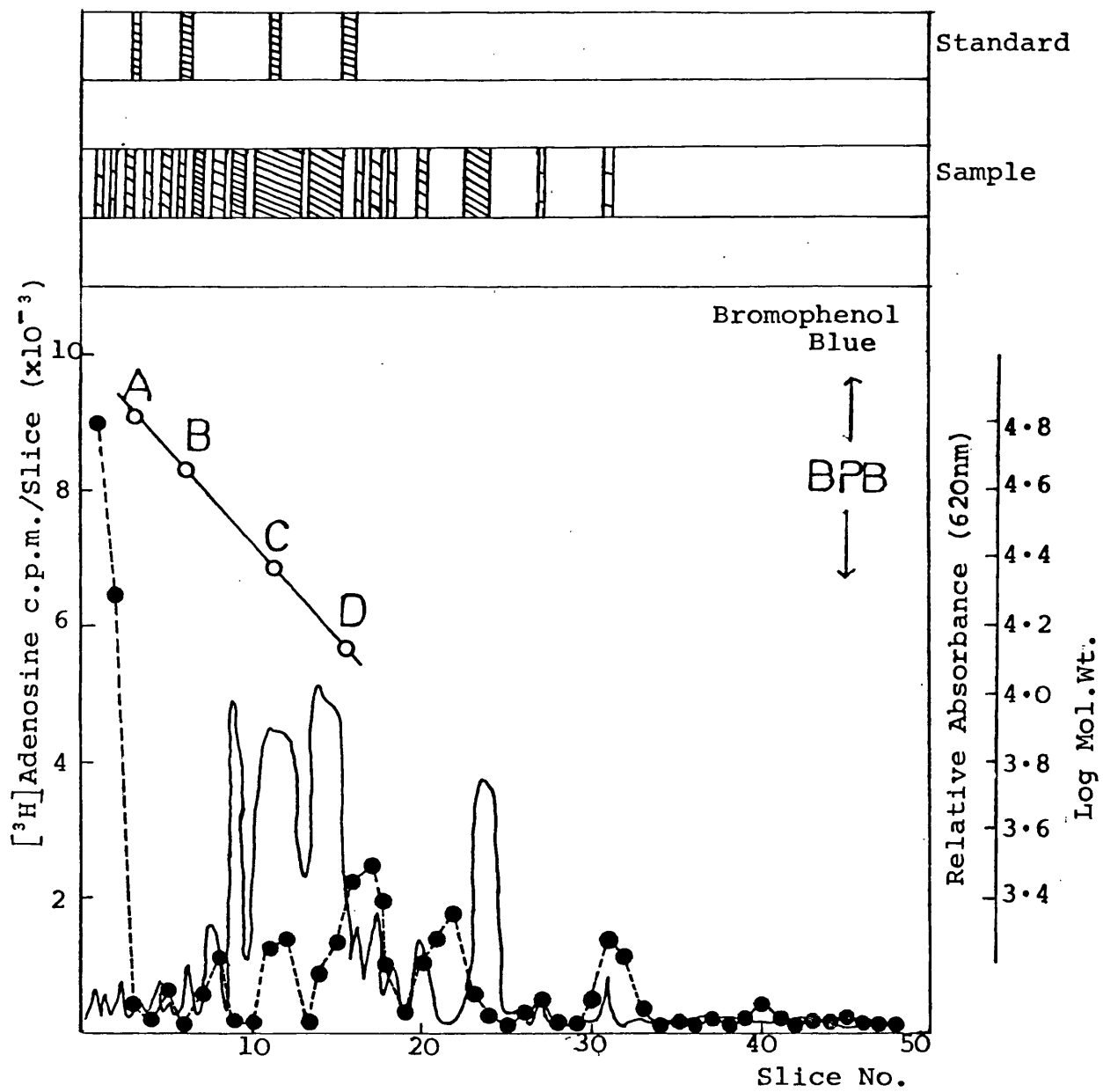


Table 4.10 Distribution of Protein and [³H]Adenosine
on SDS Polyacrylamide Gel Electrophoresis

Band Number	R _f Value	Mol.Wt.	ADP-Ribosylated [*]
1	0.02	95,500	+
2	0.04	87,000	+
3	0.06	72,500	-
4	0.08	65,000	-
5	0.10	56,000	+
6	0.11	53,000	-
7	0.12	50,000	-
8	0.17	37,000	+
9	0.19	32,500	-
10	0.24	21,000	+
11	0.29	17,500	+
12	0.33	14,000	+
13	0.35	12,000	+
14	0.37	10,500	-
15	0.42	7,500	+
16	0.48	5,000	-
17	0.56	3,000	+
18	0.65	2,000	+

* Significance of ADP-ribosylation tested using Student's t-test.

species from mouse L1210 cells are ADP-ribosylated in vivo.

4.11 DISCUSSION

Preliminary enzymic characterization of [^3H]adenosine-labelled material from the top fractions of a CsCl gradient suggested that the majority of radioactivity was nucleic acid-like material covalently bound to protein; 92% was converted to the acid-soluble form on treatment with snake venom phosphodiesterase (in the presence of urea) and 68% with Pronase. The increase in release of acid-soluble radioactivity by snake venom phosphodiesterase in the presence of urea could have been a result of one or both of the following: (i) stimulation of the enzyme by urea, (ii) increased accessibility to mono-(ADPR) or poly(ADPR) on protein. Since about 95% of radioactivity is generally converted to the acid-soluble form by snake venom phosphodiesterase in the absence of urea in vitro, it may be that some sites of ADP-ribosylation on protein in vivo differ from those in vitro. In fact, the rates of both enzymic and chemical hydrolysis of in vivo material appeared to be slower than that observed in vitro, e.g. Riquelme et al. (1979), and may also be related to the sites of ADP-ribosylation on protein in vivo compared with that in vitro. The observation is similar to that made by Young & Sweeney (1978). The release of 39% of radioactivity into the acid-soluble form on treatment with a crude preparation of poly(ADPR) glycohydrolase supported the contention

that material on top of the gradient was poly(ADPR).

However, the presence of a small amount of RNA was suggested by the results of digestion with RNase A and spleen phosphodiesterase.

T.l.c. analysis confirmed the presence of ADP-ribosylated protein; PR-AMP and 5'AMP were identified as the products of digestion by snake venom phosphodiesterase. Treatment with alkaline phosphatase dephosphorylated these products to give ribosyl-adenosine and adenosine. Furthermore, ADPR was identified as the product of digestion by crude poly(ADPR)-glycohydrolase.

Chemical hydrolysis of the [³H]adenosine-labelled material suggested that 87% was susceptible to alkali and 21% susceptible to hydroxylamine. T.l.c. analysis of the hydroxylamine hydrolysis showed that 17% of radioactivity ran as ADPR. The number of ADPR-protein linkages sensitive to base was similar to but slightly lower than that observed in vitro. The number of ADPR-protein linkages susceptible to hydroxylamine was low compared to that in vitro (Nishizuka et al., 1969; Adamietz & Hilz, 1976), but similar to those obtained by radioimmunoassay of mono(ADPR)-protein conjugates in intact EAT and rat liver cells (Bredehorst et al., 1978c) and lymphocytes (Wielckens et al., 1980). Since some ADPR-protein residues were sensitive to hydroxylamine it may be that an ester linkage exists in vivo. The low value of linkages sensitive to hydroxylamine may be related to the observation of Young & Sweeney (1978) that in vivo mono(ADP-ribosyl)ated ovum proteins appeared to be

resistant to hydroxylamine, the majority of ADP-ribosylated proteins in mouse L1210 cells were modified by a single ADPR residue.

Extensive base hydrolysis confirmed the alkali-sensitivity of the ADPR-protein linkage and the presence of a small amount of RNA. The 81.5% conversion of ADPR to 5'AMP under these conditions was close to the value observed by Goebel et al. (1977). Re-analysis of the base digest by CsCl density gradient centrifugation revealed that about 7% of total ADPR-protein linkages may be resistant to alkali. Similar observations were made by Adamietz et al. (1978), on analysing the alkali-sensitivity of histone H1 ADP-ribosylated in vivo, and Young & Sweeney (1978), on analysing the in vivo ADP-ribosylation of ovum proteins. The lack of a defined peak of radioactivity at the expected position of free poly(ADPR) on the caesium chloride gradient may have been a consequence of the short average chain length of polymer released. Even a mean chain length of 15, corresponding to a molecular weight of about 7,500, does not favour sharp banding (Adamietz & Hilz, 1976). The radioactive profile of free poly(ADPR) was in fact similar to that obtained by Adamietz & Hilz (1976) and Furneaux & Pearson (1980).

Chain length analyses of both the products of snake venom phosphodiesterase and alkaline phosphatase digests revealed an average chain length of 1.3. This value is very low compared to that obtained from in vitro analyses (e.g. Adamietz et al. (1980) but is in

agreement with a number of in vivo analyses (Ueda et al., 1975; Adamietz et al., 1978; Young & Sweeney, 1978, 1979). The possibility that the low chain length value may be attributed to poly(ADPR) glycohydrolase activity does not hold since the conditions employed throughout the isolation procedure ensured that all enzymes were inactivated. It may be that, as suggested by Farzaneh & Pearson (1978), a large proportion of ADP-ribosylation in vitro is an elongation of that previously initiated in vivo. The longer average chain lengths observed in in vitro may, in fact, be a result of the nuclear isolation procedures employed. Under these conditions the chromatin structure is often drastically altered and the DNA is probably damaged. Such conditions have been shown to cause an increase in poly(ADPR) synthetase activity (Halldorsson et al., 1978; Berger et al., 1979a) and poly(ADPR) levels (Juarez-Salinas et al., 1979; Shall et al., 1980). The ratio of mono(ADPR) to poly(ADPR) obtained by chain length analyses, was confirmed by aminoethyl cellulose chromatography of a sample previously digested with base. This method also allowed an estimation of the average chain length of poly(ADPR) to be made. A value of 2.92 was obtained which agreed well with a calculated value of 3.3.

These results confirm unambiguously the existence of mono(ADPR) and poly(ADPR) bound to proteins in vivo and also demonstrate that the CsCl density gradient method developed gave excellent separation of ADP-ribosylated proteins from DNA and RNA; only about 2% of RNA appeared

to be associated with the protein fraction.

The extensive characterization of isolated ADP-ribosylated protein enabled a reliable estimation of the percentage of acid-insoluble radioactivity present as ADPR bound to protein to be made. Total ADP-ribosylated protein accounted for 11.18% of acid-insoluble radioactivity; 3.35% was as poly(ADP-ribosyl)ated protein and 7.83% as mono(ADP-ribosyl)ated protein. Since only about one quarter of DNA and RNA are labelled by [^3H]-adenosine then these values may be reduced to account for the total potential labelling in DNA and RNA. Thus the quantity of ADPR present with respect to that of DNA and RNA is about 0.84% as poly(ADPR) and 1.96% as mono(ADPR). These values, however, are only approximate and not quantitative; no information is available on the actual levels of ADP-ribosylated protein in the cell or the different rates of incorporation or turnover of [^3H]-adenosine on DNA, RNA and ADP-ribosylated protein. Lymphoma cells have been reported to possess a high degree of ADP-ribosylation compared to that in normal cells (Wielckens et al., 1980).

Analysis of the turnover of ADPR on protein revealed an estimated half-life of about 4.5h. Under optimal conditions for synthetase activity, Hilz & Kittler (1971) suggested that the time for turnover of (ADPR)_n residues necessary for one cell duplication was about 10 minutes. The conditions in vivo, however, are not optimal for synthetase activity but are dependent on the state of the cell at the time of analysis. Furthermore, the majority

of ADP-ribosylation in vitro is by poly(ADPR); in mouse L1210 cells most proteins were ADP-ribosylated in vivo by single ADPR residues. Poly(ADPR) glycohydrolase does not cleave mono(ADPR) from protein. The 'hydrolase' which does cleave this residue from protein may remove ADPR at a fairly slow rate; the function of mono(ADPR) or short chain oligo(ADPR) may be different from that of poly(ADPR). The half-life obtained though does demonstrate that during the 18h labelling period all poly(ADPR)/mono(ADPR) present was probably labelled.

The distribution of radioactivity observed on CsCl density gradients from stationary phase cells supports the theory that poly(ADPR) and mono(ADPR) may serve different functions. The chain length of ADPR on proteins appeared to be longer in stationary phase cells, as suggested by the lower position of the peak of radioactivity at the top of the gradient. The increase in poly(ADPR) synthetase activity during the growth cycle of cells (Stone & Shall, 1973) and the maximum observed at early stationary phase (Stone & Shall, 1973; Berger et al., 1978b) suggest that more poly(ADPR) is being synthesized at this time. Since an increase in poly(ADPR) synthetase and poly(ADPR) is always observed in response to DNA fragmentation or damage (Berger et al., 1979b; Juarez-Salinas et al., 1979) poly(ADPR) may be involved in the repair of DNA, whereas mono(ADPR) may be associated with general 'housekeeping' functions.

Analysis of the number and type of proteins ADP-ribosylated in vivo revealed that a number of species were

modified. However, the use of whole cells limited the degree of detection by SDS gel electrophoresis with respect to both the amount of protein permitted per gel and the quantity of individual protein necessary for staining by Coomassie blue. A more sensitive method is clearly necessary before a complete analysis of total proteins ADP-ribosylated in vivo may be made. Preparative gel electrophoresis may prove useful in this respect.

The extensive characterization of isolated ADP-ribosylated proteins described in this chapter showed that a number of specific proteins from mouse L1210 cells (log phase) were ADP-ribosylated and that the average chain length of ADPR on these proteins was 1.3.

CHAPTER 5

THE EFFECT OF INHIBITORS
OF POLY (ADPR) SYNTHETASE
ON ADP-RIBOSYLATION IN VIVO

5.1 INTRODUCTION

The use of inhibitors of poly(ADPR) synthetase in elucidating the role of ADP-ribosylation in nuclear function has great potential. Anomalies in cellular function observed on treatment with these inhibitors may suggest that the affected processes are dependent on the ADP-ribosylation of protein.

A number of groups of compounds have been shown to inhibit poly(ADPR) synthetase in vitro, including nicotinamide and 5-methylnicotinamide (Clark et al., 1971; Preiss et al., 1971), thymidine (Preiss et al., 1971) and methylated xanthines and cytokinins (Levi et al., 1978). However, all these compounds have been shown to affect other processes (see Section 1.3). Benzamide, an analogue of nicotinamide, was first shown to be an inhibitor of the synthetase by Shall (1975). This compound has the advantage over other analogues of nicotinamide in that it cannot be incorporated into NAD^+ since it lacks the ring nitrogen possessed by nicotinamide. The inhibition observed is consequently physiologically significant. However, the extremely low solubility and hydrophobic nature of benzamide limits its use. Purnell & Whish (1980b) realized the potential of this compound and subsequently analysed the effect of various substituted benzamides (which are more soluble) on poly(ADPR) synthetase activity. They found that 3-aminobenzamide (3-AB) and 3-methoxybenzamide (3-MB) possessed greater inhibitory ability of synthetase than any other compound

so far studied. In pig thymus nuclei K_i values of about $1.8\mu\text{M}$ and $1.5\mu\text{M}$ were observed for 3-AB and 3-MB, respectively at equimolar concentration to substrate (NAD^+). In permeabilized L1210 cells the K_i value for 3-AB is about $4.4\mu\text{M}$ (Durkacz et al., 1980). The greater degree of inhibition could not be easily explained in terms of structural or charge properties of the molecule.

Although some of these compounds known to inhibit poly(ADPR) synthesis in vitro have been used to study the function of poly(ADPR) in vivo (e.g. Farzaneh et al., 1980), no direct evidence for the inhibition of the synthetase in vivo has yet been obtained. Thus, it was of considerable interest to apply the methodology developed during the course of this work to study the effect of various inhibitors of the synthetase on the ADP-ribosylation of proteins in vivo. Two inhibitors, thymidine and 3-AB, were chosen. By using thymidine a comparison of inhibition in vivo with that already observed in a number of systems in vitro could be made. With 3-AB a physiologically significant inhibition of ADP-ribosylation could be observed.

5.2 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION

With the isolation method developed it was possible to make a direct analysis of the effect of thymidine or 3-AB on ADP-ribosylation in vivo by observing the percentage of material present in the top fractions of a CsCl gradient compared to that in control cells. 5ml of cells were taken at a density of $1.5 \times 10^5/\text{ml}$ and a 100mM

solution of 3-AB or a 250mM solution of thymidine (in 50mM Tris/HCl - pH 7.1) added to give a final concentration of 2mM or 5mM, respectively. By keeping the volume of inhibitor added as low as possible any change in cell density was made negligible. After 6 hours, when cells were at a density of about 2×10^5 /ml (equal to control cells), 40 μ Ci/ml [3 H]adenosine were added and cells incubated for a further 18 hours at 37°C. During this time treated cells remained impermeable to trypan blue, as did control cells. Cells were then harvested and CsCl density gradient centrifugation carried out as described in Section 2.2.10. A representative profile of this analysis is shown in Fig. 5.2. Calculation showed that the amount of radioactivity in the top fractions of the gradient with respect to that of control cells was 65% and 68% with 3-AB-treated and thymidine-treated cells, respectively. ADP-ribosylation was inhibited only 35% and 32% by 3-AB and thymidine. This value was surprisingly low compared to that in vitro. The amount of radioactivity in the bottom fractions of the gradient was 103% and 86% of the control for 3-AB treated and thymidine-treated cells, respectively.

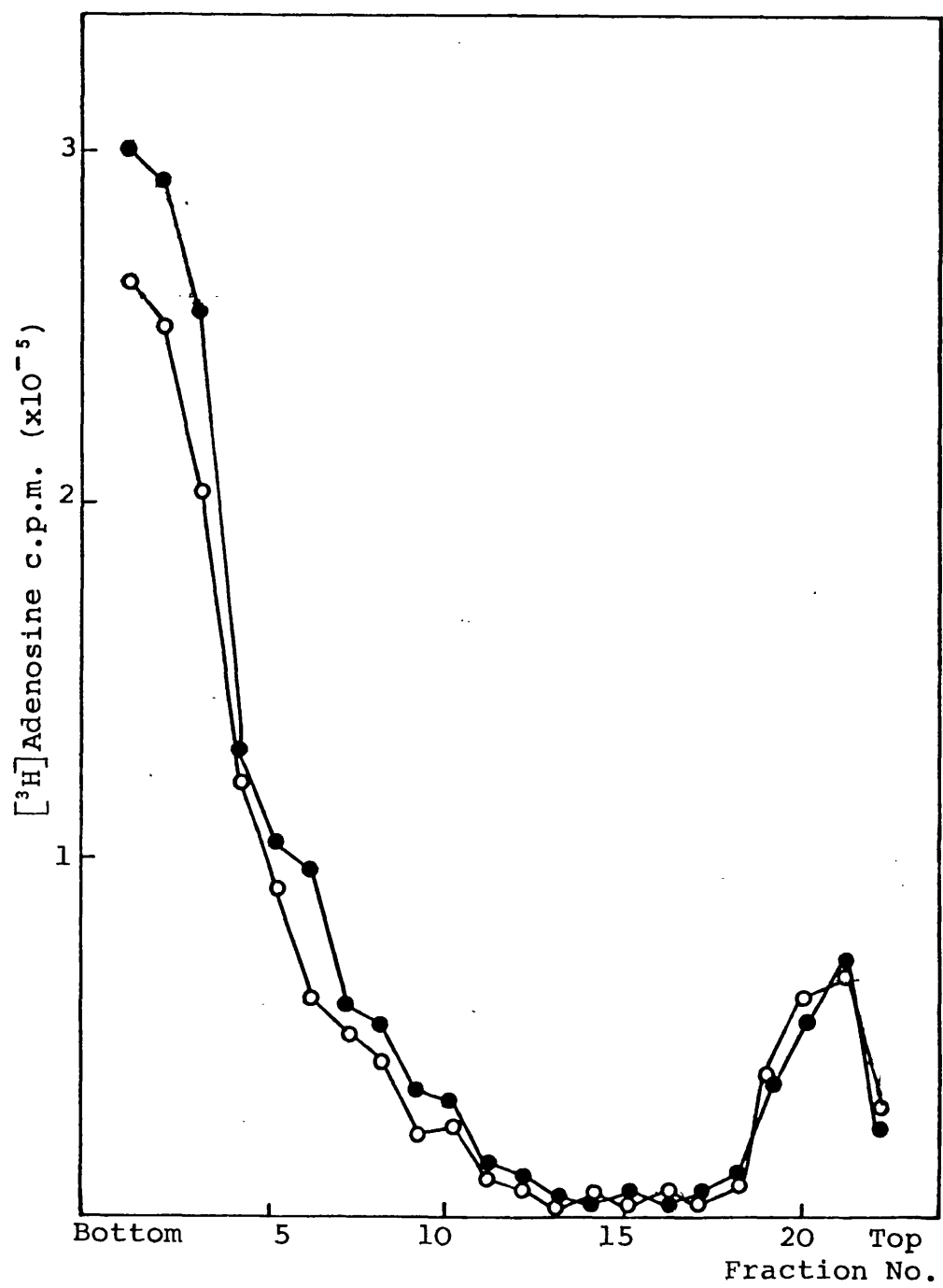
5.3 SNAKE VENOM PHOSPHODIESTERASE DIGESTION - CHAIN LENGTH ANALYSES

Since the percentage inhibition observed on CsCl density gradient centrifugation was surprisingly low, this prompted an investigation into the average chain length of the ADP-ribosylated proteins present; a

Fig. 5.2 Caesium Chloride Density Gradient Centrifugation
of Mouse L1210 Cells Treated with 3-Amino-
Benzamide or Thymidine

CsCl density gradient centrifugation was carried out as described in Section 2.2.10 and the distribution of acid-insoluble radioactivity determined (on paper discs) as described in Section 2.2.3. —●— 3-AB-treated cells, —○— thymidine-treated cells.

Fig. 5.2



differential effect on either mono(ADPR) or poly(ADPR) may have existed. Snake venom phosphodiesterase digestion was carried out as described in Section 2.2.12. An average (of three samples) of about 93% and 92% digestion was observed for 3-AB-treated and thymidine-treated cells, respectively, as determined by decrease in acid-insoluble radioactivity with time.

T.l.c. analysis of this digest, using the 1M HAc/0.9M HAc-0.3M LiCl system of Randerath & Randerath (1965a) (see Fig. 14 of the Appendix for a representative profile) showed that for 3-AB-treated cells 84% of radioactivity ran as 5'AMP and 10% as PR-AMP. For thymidine-treated cells 82% of radioactivity ran as 5'AMP and 12% as PR-AMP. Calculation of the average chain length, according to the method of Nishizuka et al. (1969), gave values of 1.12 and 1.15 for 3-AB-treated and thymidine-treated cells, respectively.

5.4 BASE HYDROLYSIS-ANALYSIS OF MONOMER/POLYMER RATIO AND AMOUNT OF RNA PRESENT

As with control cells some of the 5'AMP produced on snake venom phosphodiesterase digestion may have been attributable to RNA. This prompted an analysis by base hydrolysis. Hydrolysis was carried out as described in Section 2.2.12. An average (of three samples) of about 84% of acid-insoluble material was converted to the acid-soluble form, as determined by decrease in acid-insoluble radioactivity with time. The products of this hydrolysis were then analysed by the borate t.l.c. system of Schwartz

& Drach (1975). Analysis of the distribution of radioactivity (see Fig.15 of Appendix) revealed that for the 3-AB-treated sample 16% remained on the origin, 50% ran as 5'AMP and 33.5% ran as 3'AMP. For the thymidine-treated sample 17.5% remained on the origin, 49.5% ran as 5'AMP and 32% ran as 3'AMP. This meant that about 40% and 39% of the AMP produced by base hydrolysis of 3-AB-treated and thymidine-treated samples, respectively, was derived from RNA. A comparison of the percentage of AMP produced on snake venom phosphodiesterase digestion with that produced on base hydrolysis permitted the amount of AMP attributable to poly(ADPR), and subsequently the true average chain length of ADPR bound to proteins, to be determined. This gave a value of 1.19 for 3-AB-treated cells and 1.21 for thymidine-treated cells. Because of the reduction in ADP-ribosylated protein in the presence of inhibitors, the relative percentage of RNA present at the top of the gradient was greater than that in control cells. Correction of the average chain length for RNA subsequently showed that the value was not in fact as reduced as had originally seemed apparent, although it was slightly lower than that in control cells. About 8% of acceptor sites were occupied by poly(ADPR) in inhibitor-treated cells compared to about 11% in control cells. 3-AB and thymidine did not seem to selectively inhibit either mono(ADP-ribosylation) or poly(ADP-ribosylation). Because 15% of radioactivity on top of the gradient in control cells was attributable to RNA, then the percentage inhibition observed must be adjusted

to account for this. Correction for this showed that 41% of ADP-ribosylation was inhibited by 3-AB and 38% by thymidine.

5.5 AMINOETHYL CELLULOSE COLUMN CHROMATOGRAPHY

5.5.1 Chromatography

An alternative method by which to analyse the composition of the products of base hydrolysis was by aminoethyl cellulose chromatography. This method also permitted an average chain length analysis of poly(ADPR) alone to be made. Chromatography was carried out as described in Section 2.2.17. Representative profiles of radioactivity eluted during chromatography of 3-AB-treated and thymidine-treated samples are shown in Fig. 5.5.1. For 3-AB-treated cells 84% of radioactivity eluted in the 6M HAc fraction and 16% in the 1M ethylamine fraction. For thymidine-treated cells 82.5% of radioactivity eluted in HAc and 17.5% in ethylamine.

5.5.2 T.l.c. Analysis of 6M Acetic Acid Fraction

The lyophilized 6M HAc fraction was then subjected to borate t.l.c. so that the percentage of radioactivity attributable to mono(ADPR) or RNA could be determined. Representative profiles are shown in Fig.16 of the Appendix. For 3-AB-treated cells 12% of radioactivity ran as ADPR, 47% as 5'AMP and 40% as 3'AMP. For thymidine-treated cells very similar values were obtained, 12% of radioactivity ran as ADPR, 48% as 5'AMP and 39% as 3'AMP. An average (of

Fig. 5.5.1 Aminoethyl Cellulose Chromatography of
Base Digest of Cells Incubated with
3-Aminobenzamide or Thymidine

The lyophilized ethylamine digest was dissolved in 100 μ l of distilled H₂O and 50 μ l were applied to an aminoethyl cellulose column (2cm x 0.785cm). The sample was eluted by stepwise application of 6M HAc, distilled H₂O and 1M ethylamine as described in Section 2.2.17. The distribution of radioactivity was determined as described in Section 2.2.3. A - 3-AB, B - Thymidine.

Fig. 5.5.1A

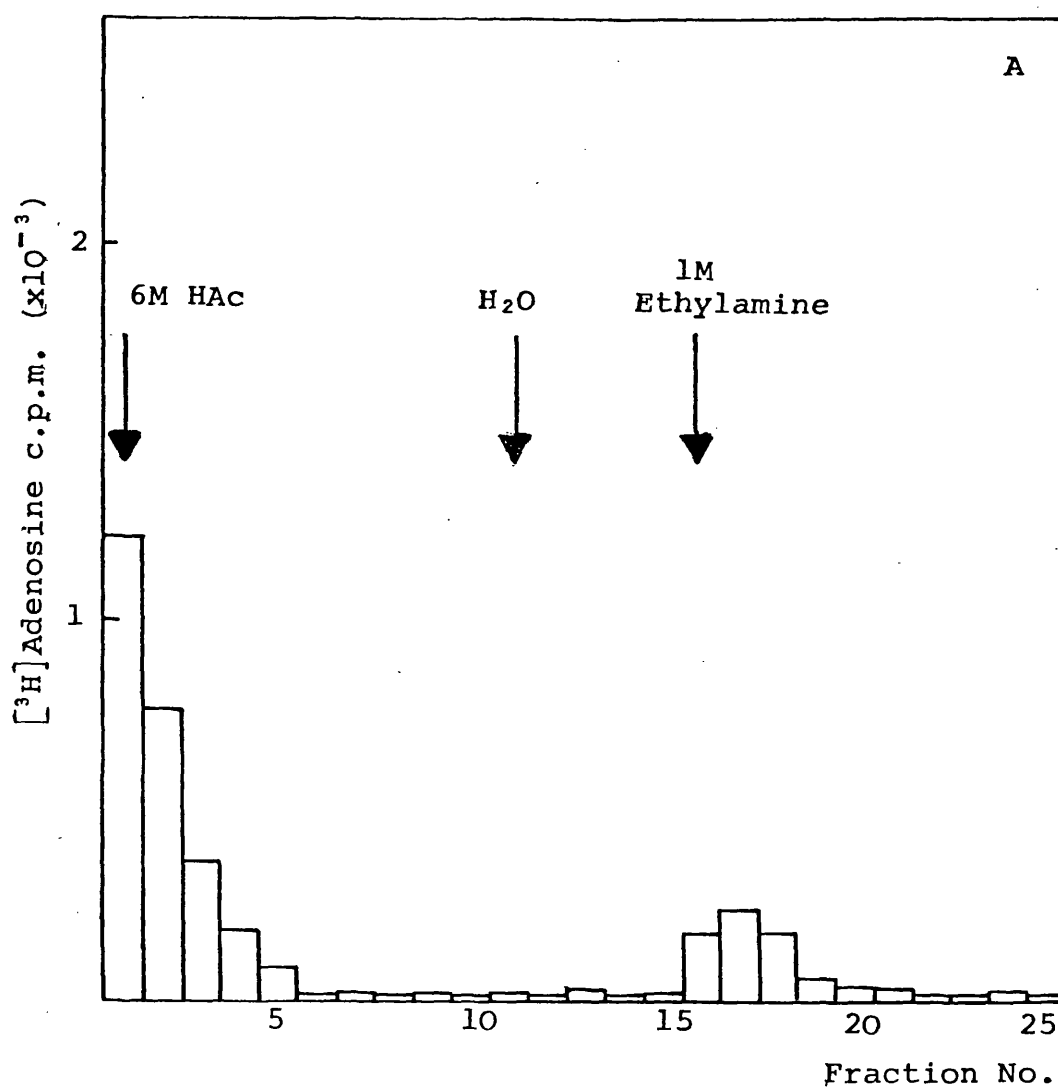
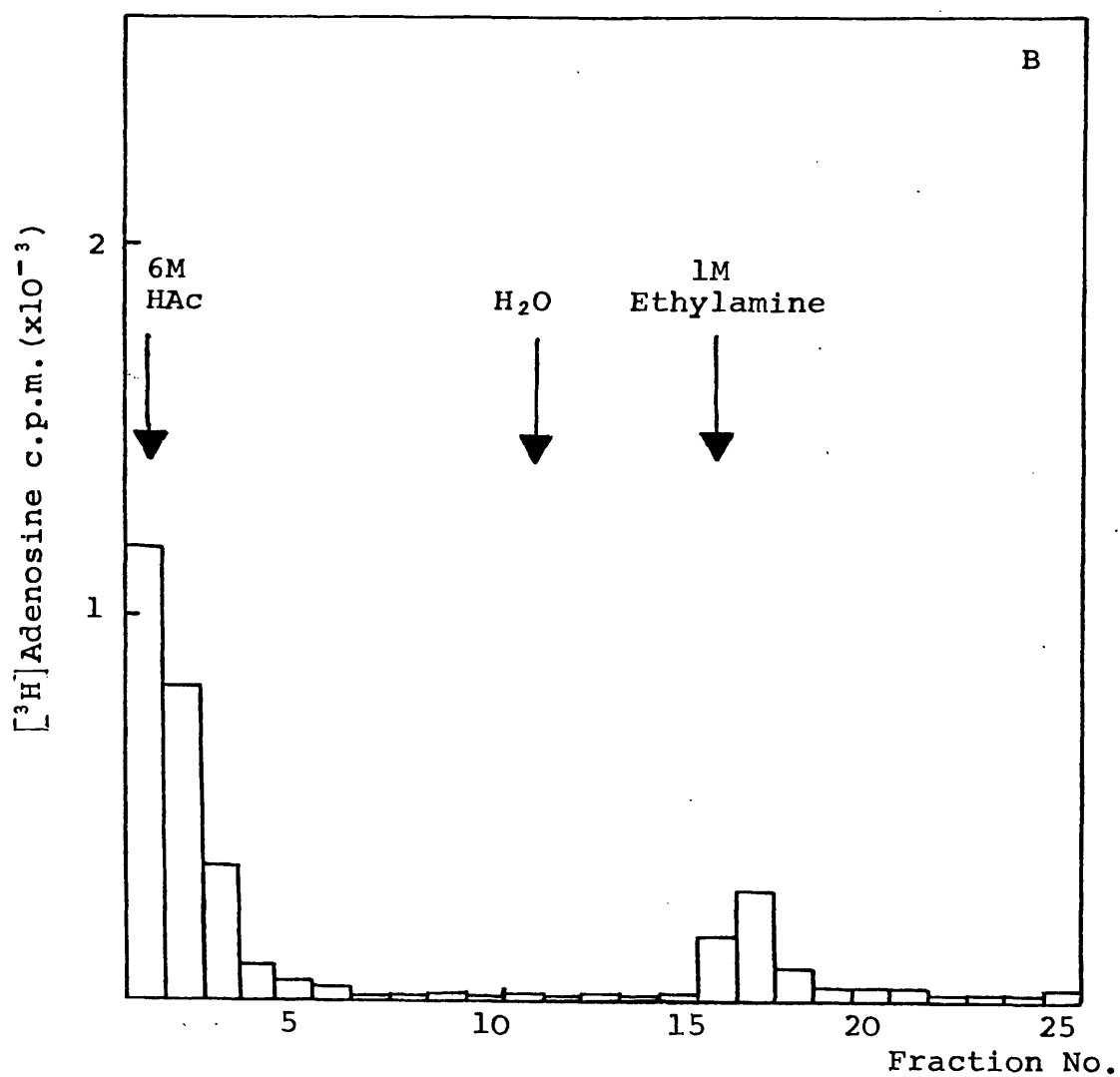


Fig. 5.5.1B



six samples) of 83% of ADPR was converted to 5'AMP during treatment with base overnight, which agreed well with the value obtained in control cells.

5.5.3 Snake Venom Phosphodiesterase Digestion of 1M Ethylamine Fraction

Snake venom phosphodiesterase digestion of the lyophilized ethylamine fraction from aminoethyl cellulose chromatography converted 94% of the radioactivity into the acid-soluble form, as determined by decrease in acid-insoluble radioactivity with time.

T.l.c. analysis of the products of digestion, using the 1M HAc/HAc-LiCl system of Randerath & Randerath (1965a) (see Fig. 17 of Appendix) revealed that for 3-AB treated cells 61% of radioactivity ran as PR-AMP and 32% as 5'AMP. For thymidine-treated cells 65% ran as PR-AMP and 30% as 5'AMP. Thus, the average chain length of poly(ADPR) in 3-AB-treated and thymidine-treated cells was 2.90 and 3.17, respectively. These values agree well with calculated values of 3.02 and 3.07.

5.6 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF CELLS TREATED FOR 6 HOURS WITH INHIBITORS OF POLY(ADPR) SYNTHETASE

It was interesting that although both thymidine and 3-AB were very good inhibitors in vitro they were only capable of producing about 40% inhibition in vivo, even at concentrations a thousand times that required for half-maximal inhibition in vitro (Purnell & Whish, 1980b) and five hundred times that in permeabilized cells (Durkacz

et al., 1980). The addition of thymidine is known to affect the synthetic pathways of various nucleotides, in particular it depletes dCTP levels in the cell (Meuth et al., 1976) and subsequently inhibits DNA synthesis (Bjursell & Reichard, 1973). This may affect the inhibition of ADP-ribosylation in vivo in some way; the suppression of DNA synthesis has in fact been shown to cause an increase in poly(ADPR) synthetase activity (Berger et al., 1978a). 3-AB, however, cannot be metabolized by NAD⁺ biosynthetic enzymes and does not appear to affect any metabolic pathways other than the synthesis of poly(ADPR). Furthermore, t.l.c. analysis revealed that 3-AB remained unmodified even after 72 hours in Burkitt's lymphoma cells (Daudi) (Purnell & Whish, 1977).

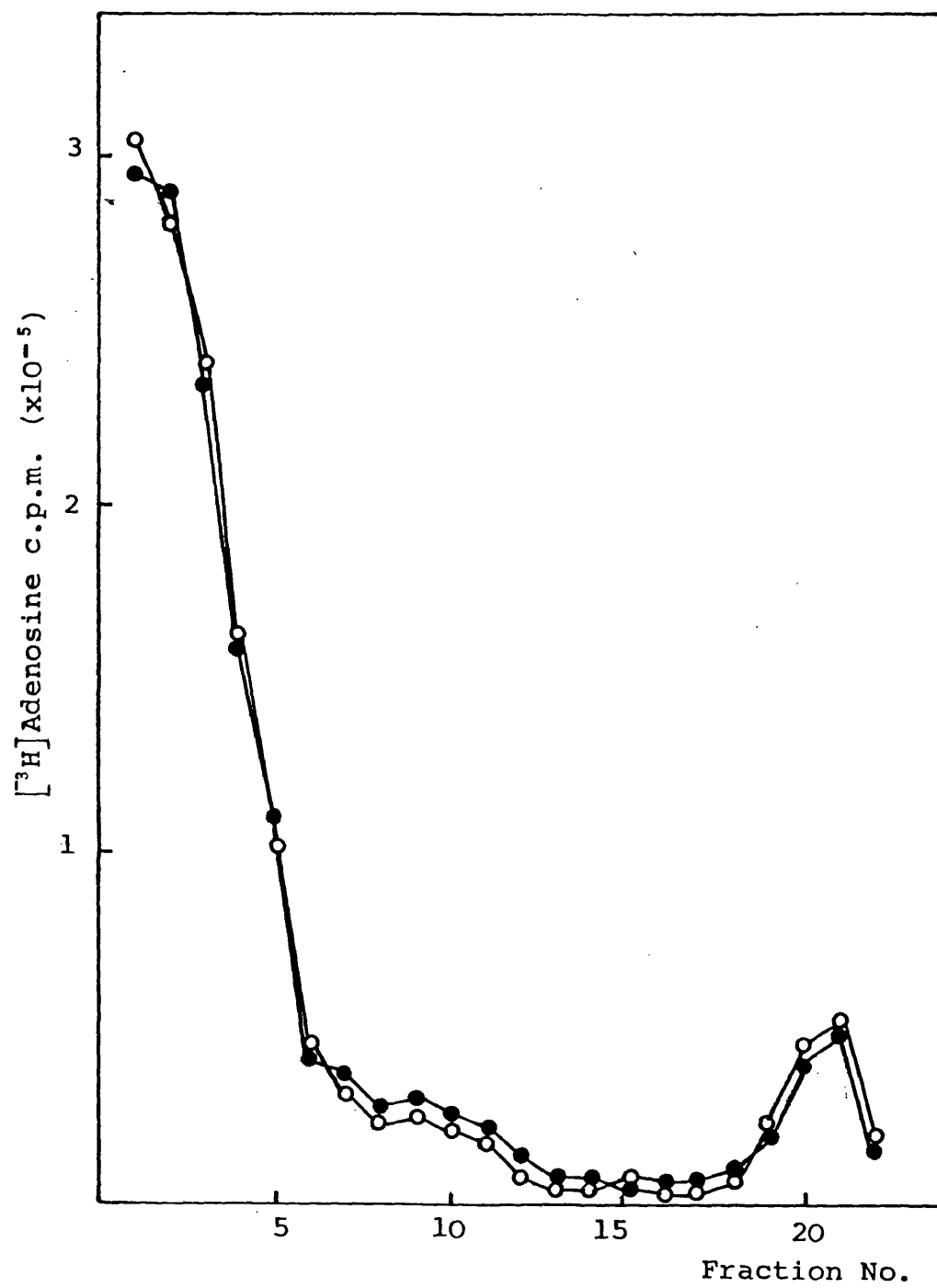
The low values of inhibition observed in cells treated for 24 hours with these inhibitors prompted a re-analysis by determining the extent of ADP-ribosylation in vivo after a much shorter period of treatment. During this re-analysis thymidine treatment was carried out in the presence of 2'deoxyctidine in an attempt to produce a more physiologically significant inhibition.

Thus, cells were treated with either 2mM 3-AB or 2mM thymidine/1mM 2'deoxyctidine for 6 hours. 40µCi/ml [³H] adenosine were added 5 minutes after addition of inhibitor. Cells were harvested and CsCl density gradient centrifugation carried out as described in Section 2.2.10. A representative profile of the distribution of radioactivity on centrifugation is shown in Fig. 5.6. The percentage of radioactivity compared with that in control

Fig. 5.6 Caesium Chloride Density Gradient
Centrifugation of L1210 Cells Treated
with 3-Aminobenzamide or Thymidine/
Deoxycytidine for 6 Hours

Caesium chloride density gradient centrifugation was carried out as described in Section 2.2.10 and the distribution of radioactivity determined (on paper discs) as described in Section 2.2.3. —●— 3-AB-treated cells, —○— thymidine-treated cells.

Fig. 5.6



cells was 45% for 3-AB-treated cells and 48% for thymidine-treated cells. 55% and 52% of ADP-ribosylation is inhibited by 3-AB and thymidine. Correcting for RNA, 65% inhibition was observed on treatment for 6 hours with 3-AB and 62% with thymidine. The degree of inhibition observed after a 6 hour treatment was greater than that after a 24 hour treatment. This suggests that the cells might in some way be adjusting to the presence of these inhibitors.

5.7 DISCUSSION

ADP-ribosylation of proteins in vivo was inhibited in the presence of 3-AB or thymidine. The inhibition was not, however, complete, at least at the times chosen for analysis. After a 24 hour incubation with inhibitors the ADP-ribosylation observed on CsCl density gradient centrifugation was 59% and 62% of that of control cells for 3-AB and thymidine, respectively. Subsequent chain length analysis revealed an average value of 1.19 for 3-AB-treated cells and 1.21 for thymidine-treated cells. This is the first chain length analysis to be carried out on total ADP-ribosylated proteins in vitro or in vivo after treatment with inhibitors of poly(ADPR) synthetase.

The number of acceptor sites occupied by poly(ADPR) was 8% compared with 11% in control cells. Although preliminary chain length analysis suggested an apparent selective inhibition of poly(ADPR), correction for RNA present showed that the chain length did not in fact

differ much from that in control cells. The average chain length of poly(ADPR) was 2.90 for 3-AB-treated and 3.17 for thymidine-treated cells. Analysis of ADP-ribosylation after only 6 hours in the presence of inhibitors revealed that the degree of modification was 35% and 38% of that in control cells for 3-AB and thymidine, respectively. This suggested that the degree of inhibition decreased with incubation time.

The inhibition of ADP-ribosylation in vivo appears to differ from that observed in vitro. It is known that the degree of ADP-ribosylation in vitro far exceeds that in permeabilized cells, which more closely reflect the situation in vivo, and may be a result of the nuclear isolation procedures employed (Halldorsson et al., 1978). It is possible that this increase in ADP-ribosylation is attributable to a specific fraction of ADP-ribosylated protein in response to, for example, DNA damage. Indeed an increase in poly(ADPR) rather than mono(ADPR) has been shown to occur under these conditions (Berger et al., 1979a; Juarez-Salinas et al., 1979). Furthermore, since the amount of ADP-ribosylated protein sensitive to hydroxylamine in vitro exceeds that observed in vivo (Young & Sweeney, 1978; Adamietz et al., 1978), the increase may be attributable to a hydroxylamine-sensitive poly(ADPR)-protein fraction. However, to date no attempt has been made to determine to what extent any ADP-ribosylation occurs in vitro in the presence of inhibitors of poly(ADPR) synthetase or the characteristics of this ADP-ribosylation, if any.

Since this study was undertaken a number of reports have been made on the effect of inhibitors of poly(ADPR) synthetase on in vivo ADP-ribosylation. Ferro et al. (1978) showed that nicotinamide injected into rats increased several-fold the amount of (ADPR)_{n>4} in the liver. Although stimulation of ADP-ribosylation rather than inhibition was observed, they did suggest that inhibition might have occurred immediately after injection but that this rapidly diminished as the concentration of nicotinamide in the cell decreased. Bredehorst et al. (1980) showed that an increase in mono(ADPR)-protein conjugates also occurred on nicotinamide administration and that hydroxylamine-sensitive ADPR-protein conjugates were significantly more affected than hydroxylamine-resistant conjugates. Berger & Sikorski (1980a) demonstrated an intriguing effect of nicotinamide on the response to DNA damage in the cell. Nicotinamide stimulated DNA repair synthesis, probably through poly(ADP-ribosyl)ation, in DNA-damaged cells but inhibited synthesis in cells not subjected to DNA damage. The concentration of nicotinamide used, 2-5mM, was comparable to that able to inhibit poly(ADPR) synthesis in vitro. Apparently, conditions in vivo favour the incorporation of nicotinamide into NAD⁺ rather than the inhibition of poly(ADPR) synthetase. Tanuma et al. (1979) showed that thymidine or nicotinamide administered in vivo resulted in an increase in poly(ADP-ribosyl)ation in vitro, probably by an increase in acceptor sites.

The inhibition of ADP-ribosylation observed in mouse

L1210 cells on treatment with 3-AB or thymidine may be a result of two alternatives. It may be that total inhibition did occur soon after addition of 3-AB or thymidine and that the incomplete inhibition observed was a result of some adaptation by the cell to the situation with time. The fact that the degree of inhibition decreased with time supports this theory. Alternatively, the situation may have been much more complex. It may be that a combination of inhibition and stimulation occurred on addition of 3-AB or thymidine. For example, an increase in acceptor sites for ADP-ribosylation may have 'masked' the extent to which inhibition of ADP-ribosylation did occur. Furthermore, no information is available on the effect of these inhibitors on NAD^+ levels in the cell. The effect of these inhibitors may then have decreased with time as the cells adjusted to their presence or as the compounds were metabolized. Although 3-AB has been shown to remain unmodified even after 72 hours, it is not known whether the concentration of this inhibitor in the cell decreases with time (i.e. no quantitative analysis has yet been made) or indeed how much of the inhibitor is in association with the chromatin compared with that originally added to the cell.

These studies are the first to demonstrate the specific inhibition of ADP-ribosylation in vivo. The inhibition appeared to be more complex than that in vitro. The decrease in inhibition observed with time is consistent with the results of Whish (personal communication) who showed that cells are still able to

grow in the presence of 3-AB for substantial periods of time; it seems that cells can adjust to the presence of inhibitors of poly(ADPR) synthetase.

The effect of inhibitors of poly(ADPR) synthetase on ADP-ribosylation in vivo is still unclear at present. In order to obtain a more complete picture it will be necessary to analyse ADP-ribosylation soon after addition of these inhibitors and also to characterize the ADP-ribosylation of proteins in their presence. The present analysis has demonstrated, though, that in order to determine the true function of ADP-ribosylation it is necessary to study the process in vivo.

CHAPTER 6

THE EFFECT OF
DIMETHYL SULPHATE ON
ADP-RIBOSYLATION IN VIVO

6.1 INTRODUCTION

During recent years the most predominant and consistent observation made during the study of ADP-ribosylation is that treatment of nuclei or cells with DNA damaging agents results in a rapid lowering of NAD^+ levels and a concomitant increase in poly(ADPR) synthetase activity and levels of poly(ADPR). Since the drop in NAD^+ was demonstrated not to be a result of inhibition of synthesis (Whish et al., 1975), of degradation by NAD^+ glycohydrolase (Skidmore et al., 1979; Jacobson et al., 1980) or of turnover of NAD^+ after a decrease in rate of synthesis (Hilz et al., 1963), it seems that a direct correlation exists between the NAD^+ drop and the increased poly(ADPR) synthetase activity. The increased activity may be brought about by either alkylating agents, irradiation or endonucleases (see Section 1.8.6). The common effect of all these agents is that they ultimately cause DNA strand breakage, suggesting that poly(ADPR) may have a role in DNA repair. This hypothesis is supported by the finding that addition of inhibitors of poly(ADPR) synthetase markedly increases the cytotoxicity of the DNA damaging agents (Davies et al., 1978; Goodwin et al., 1978) and also prevents rejoining of DNA strand breaks (Durkacz et al., 1980). The same effect was observed on depleting cells of NAD^+ by growing in nicotinamide-free medium (Jacobson & Narasimhan, 1979; Durkacz et al., 1980). Furthermore, XP cells which are unable to excise pyrimidine dimers, are deficient or delayed in this response (Berger

et al., 1980a). The response seems to be very rapid; on x-irradiation a cell can consume more than 90% of its NAD⁺ in less than 5 minutes (Benjamin & Gill, 1980). The role which poly(ADPR) plays in DNA repair is, however, still uncertain at present. It is known, though, that the increase in poly(ADPR) occurs at a point distal to DNA strand breakage (Farzaneh et al., 1980) but before repair synthesis (Berger & Sikorski, 1980b).

The increase in poly(ADPR) levels on treatment with DNA damaging agents has generally been analysed in permeabilized cells. Although such cells provide a good reflection of the situation in vivo and have proved extremely useful in the study of ADP-ribosylation, it may still be that ADP-ribosylation observed under these conditions differs markedly from that in vivo. The development of a method for the isolation of protein ADP-ribosylated in vivo during the course of this work thus provided an opportunity to analyse for the first time the effect of DNA damaging agents on ADP-ribosylation in vivo. Furthermore, since the protein-ADPR bond remains intact during isolation, any increase in ADP-ribosylation observed will be a result of protein-bound poly(ADPR). The method potentially allows the identification of any protein or proteins specifically ADP-ribosylated in response to DNA damage and may therefore provide a major contribution towards the elucidation of the role of ADP-ribosylation in nuclear function. The effect of DMS on ADP-ribosylation in vivo is described in the next section.

6.2 CAESIUM CHLORIDE DENSITY GRADIENT CENTRIFUGATION OF L1210 CELLS TREATED WITH 105 μ M DMS

A simple and direct means by which to analyse the response of the poly(ADPR) system to DMS in vivo was to determine the percentage of [3 H]adenosine-labelled material on top of a CsCl gradient compared with that in control cells. Cells were first labelled for 18h with 40 μ Ci/ml [3 H]adenosine as normal. DMS was then dissolved in distilled water at a ratio of 1:19 (by vol.) and 1 μ l samples of this solution rapidly transferred to 5ml of [3 H]adenosine-labelled L1210 cells at 37°C, to give a final concentration of DMS of 105 μ M. This concentration of DMS was chosen because it had previously been shown to produce maximum lowering of NAD $^+$ levels from which the cell could still recover with time (Durkacz et al., 1980). At various time points after addition of DMS the cells were harvested as described in Section 2.2.9 and CsCl density gradient centrifugation carried out as outlined in Section 2.2.10. The distribution of radioactivity observed after centrifugation is shown in Fig.6.2. At one minute after the addition of DMS the distribution of radioactivity on top of the gradient was essentially the same as that in control cells i.e. 0 minutes. At 2 minutes and 5 minutes, however, the extent and distribution of radioactivity differed significantly from that in control cells. The amount of radioactivity had increased over 1.5 times that in control cells and, furthermore, the peak of radioactivity had shifted to a greater density. This shift in buoyant density was similar to that observed in stationary

Fig. 6.2 : Effect of 105 μ M DMS on the Extent of ADP-
Ribosylation in Mouse L1210 Cells

Caesium chloride density gradient centrifugation was carried out as described in Section 2.2.10 and the distribution of acid-insoluble radioactivity determined (on paper discs) as described in Section 2.2.3.

Fig. 6.2A

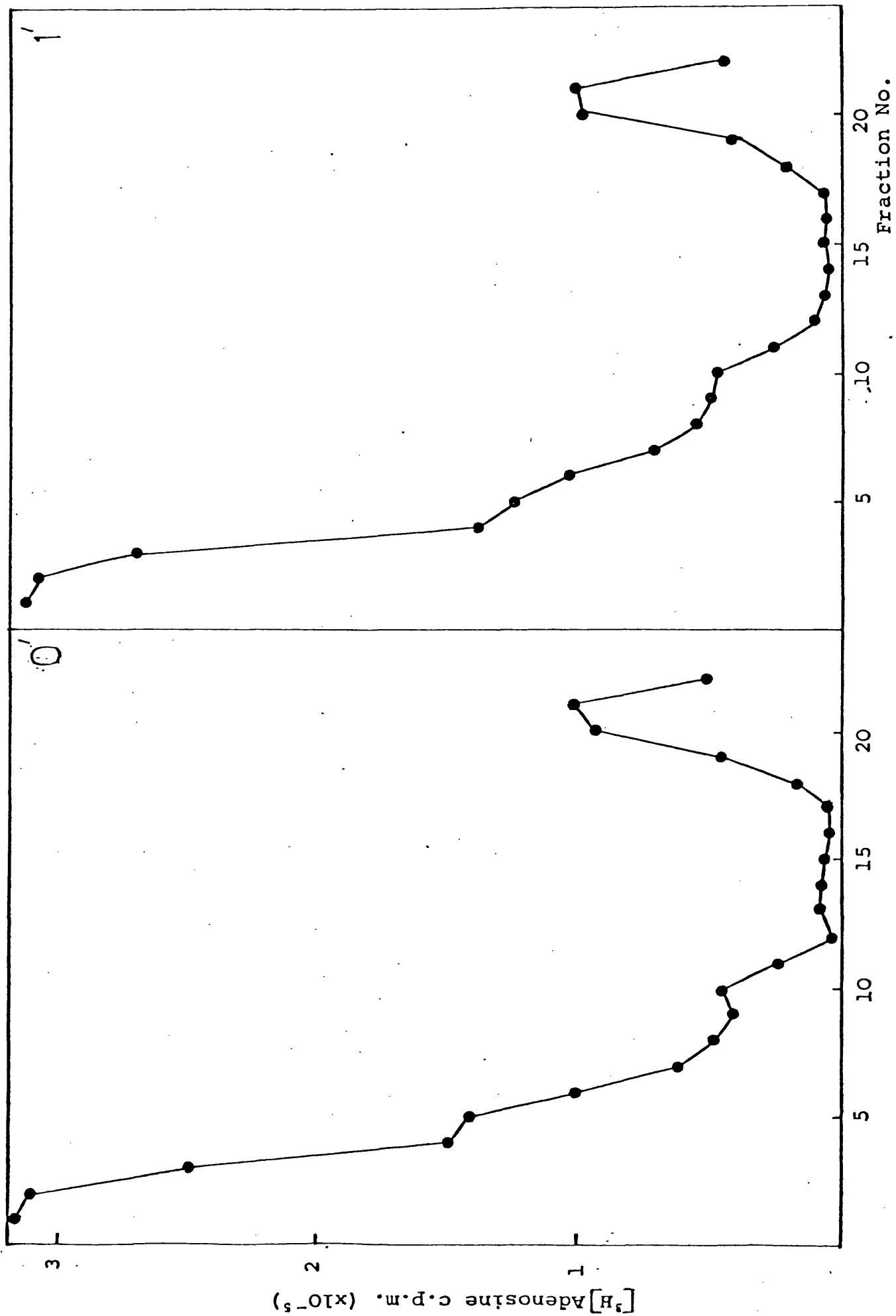


Fig.6.6.2B

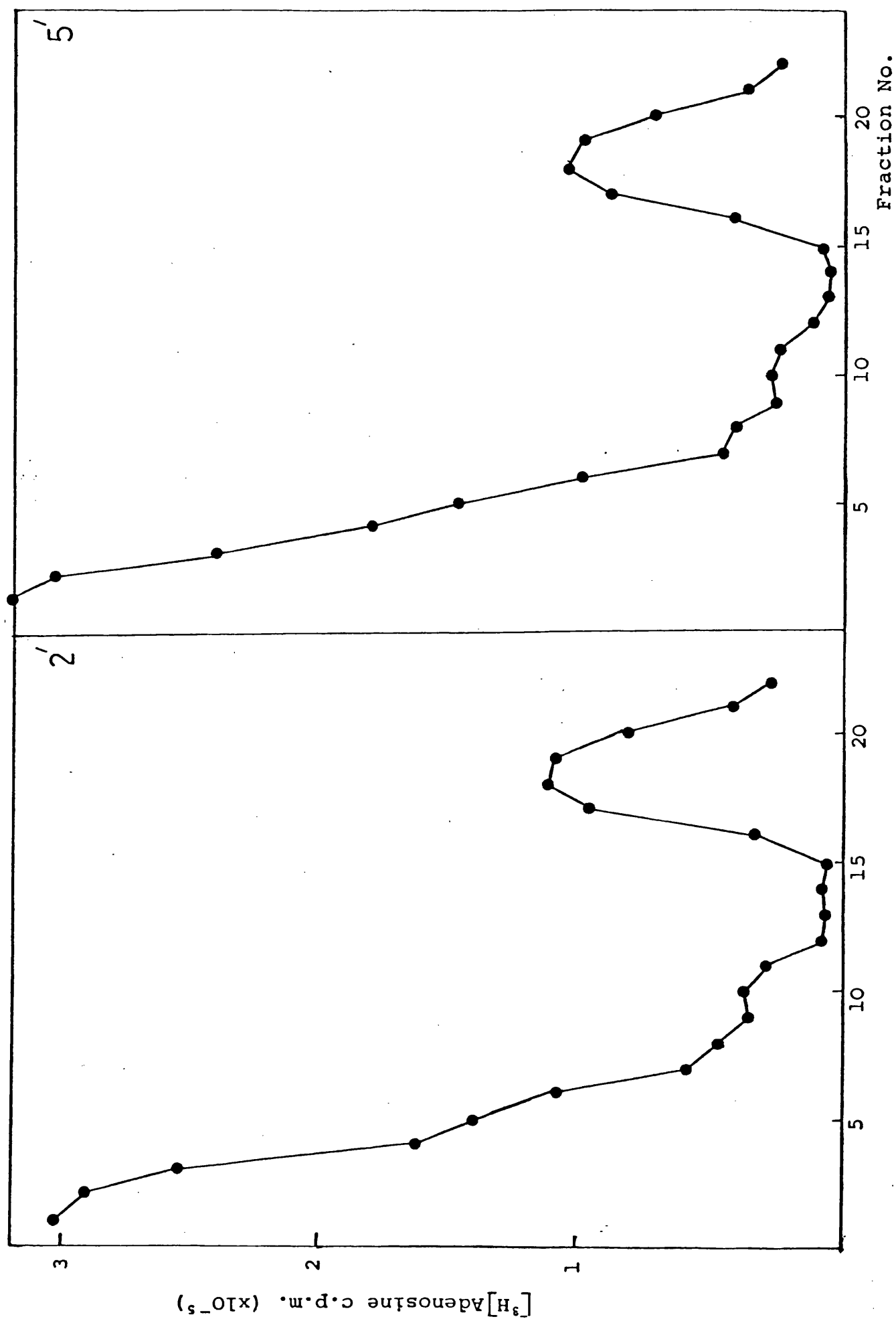


Fig. 6.2C

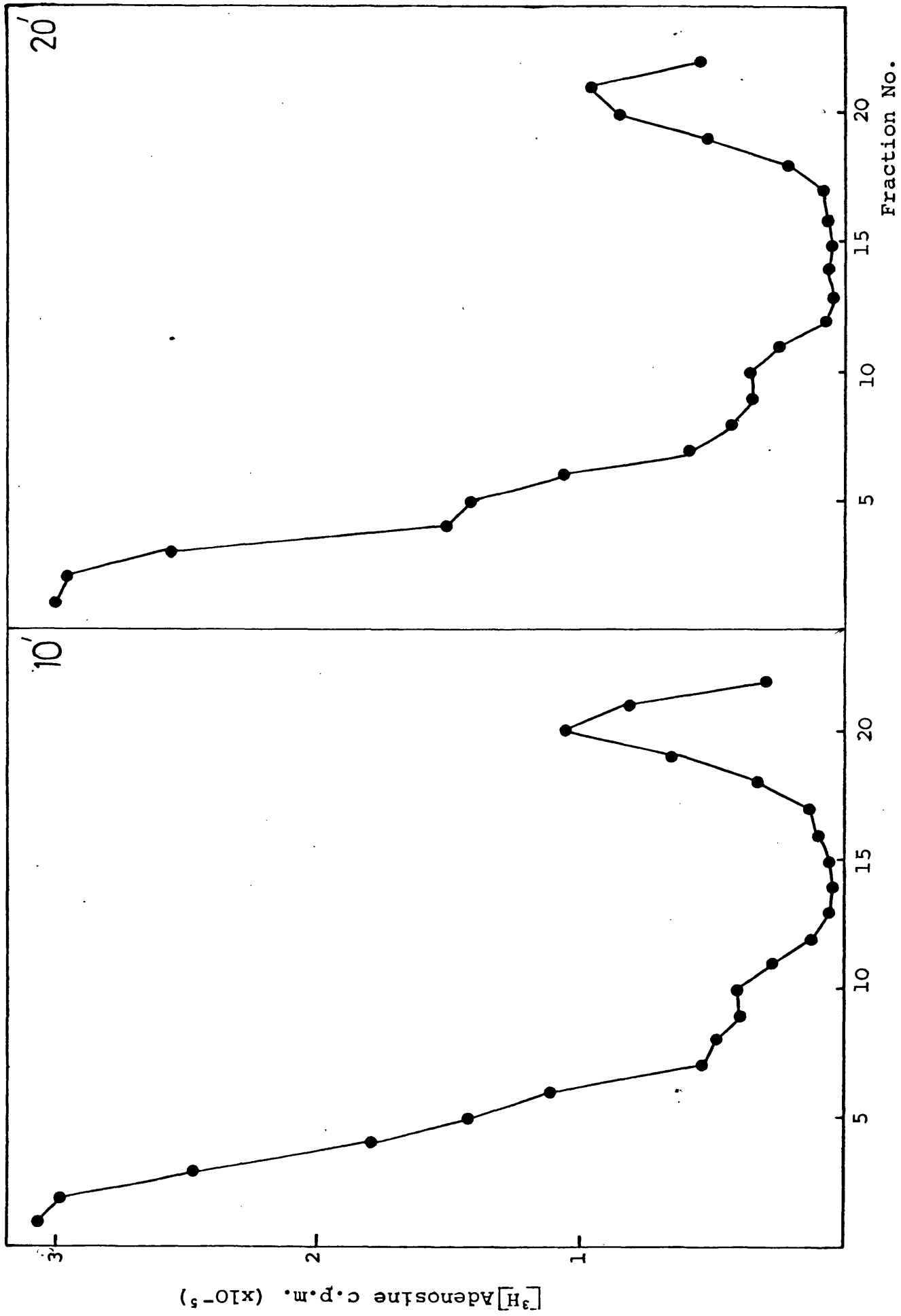
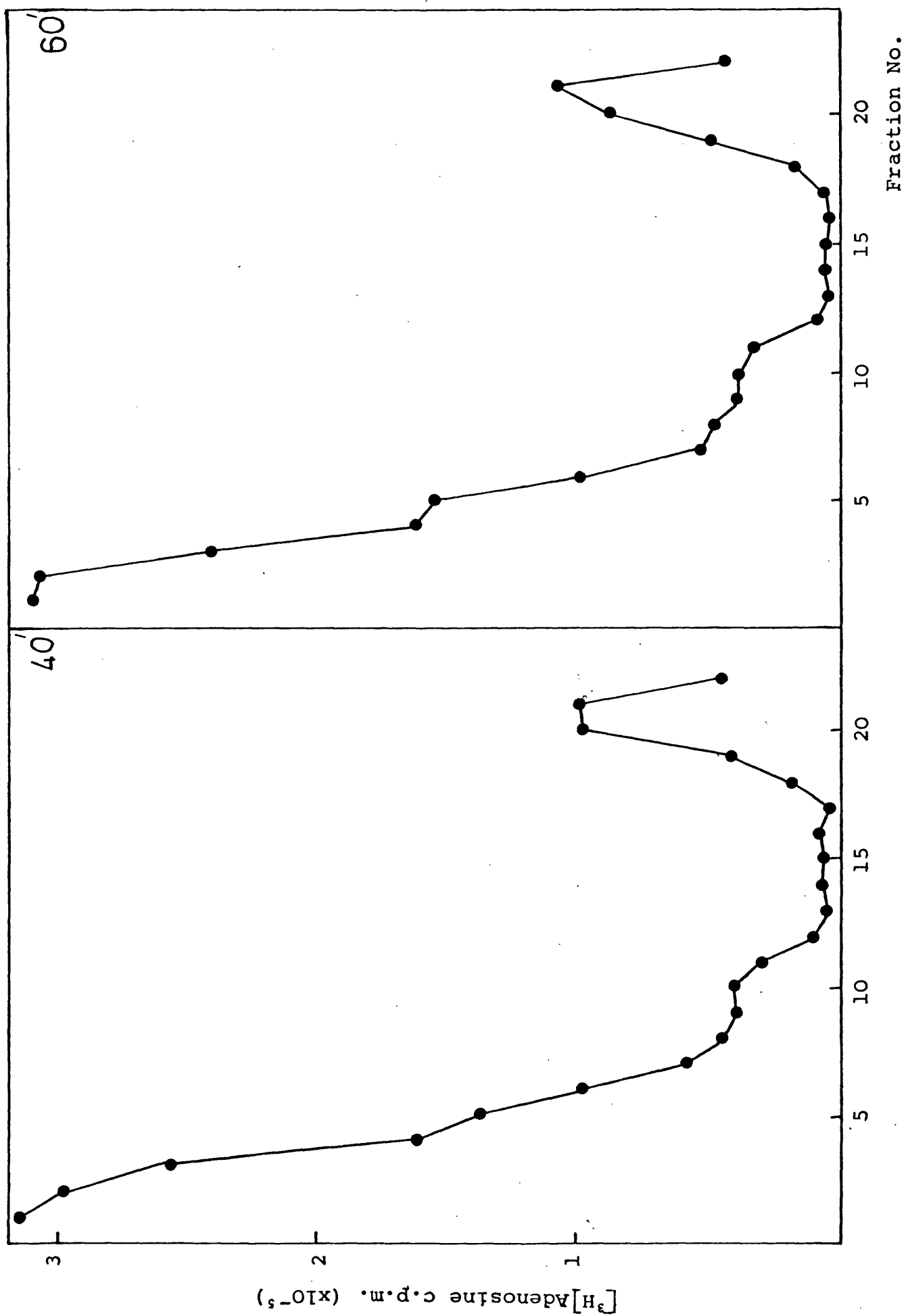


Fig. 6.2D



phase cells (see Chapter 4) and suggested that the chain length of poly(ADPR) bound to protein was greater than that in control cells. Thus, at 2 minutes after addition of DMS the average percentage of radioactivity at the top of the gradient with respect to control was 157% and that at 5 minutes was 148%. A comparison of acid-insoluble material incorporated into the top fractions of the gradient as a percentage of the total showed that 19.3% and 18.4% was incorporated at 2 and 5 minutes, respectively, compared to 13.2% in control cells. At 10 minutes after addition of DMS the observed increase in radioactivity was less pronounced; the percentage incorporated with respect to that of control cells was 106% and the peak of radioactivity, although not quite at a position equivalent to that in control cells, appeared to be approaching it. This subsequent decrease in incorporation was verified by analysis of the distribution of radioactivity after treatment with DMS for 20 minutes; the profile was essentially the same as that in control cells. At 40 minutes and 1 hour the distribution of radioactivity was also the same as that in control cells.

The increase in ADP-ribosylation observed on DMS treatment was not as dramatic as expected, although the response to the treatment was very rapid. The results of this experiment are summarized in Table 6.2. Characterization of gradient material described in Chapter 4 revealed that about 15% of acid-insoluble radioactivity at the top of the gradient was as RNA. Thus, correcting for this the stimulation of ADP-ribosylation by DMS was a little

Table 6.2 Effect of Treatment with 105 μ M DMS on the Extent of ADP-Ribosylation in Ll210 Cells as Analysed by Caesium Chloride Density Gradient Centrifugation

Duration of DMS Treatment (Min)	c.p.m. on top of Gradient	% Control
0	$3.09 \times 10^5 \pm 1.13 \times 10^3$	100.0
1	$3.07 \times 10^5 \pm 1.08 \times 10^3$	99.5
2	$4.85 \times 10^5 \pm 1.58 \times 10^3$	157.0
5	$4.57 \times 10^5 \pm 1.30 \times 10^3$	148.0
10	$3.27 \times 10^5 \pm 1.05 \times 10^3$	106.0
20	$3.10 \times 10^5 \pm 1.32 \times 10^3$	100.5
40	$3.06 \times 10^5 \pm 1.21 \times 10^3$	99.0
60	$3.12 \times 10^5 \pm 1.18 \times 10^3$	101.0

Values are the mean of 3 samples and the error represented is the standard error of that mean (S.E.M.).

higher than had previously seemed apparent; the percentage radioactivity incorporated at 2 and 5 minutes was 167% and 156% of the control, respectively.

6.3 NAD⁺ ASSAY OF MOUSE L1210 CELLS TREATED WITH 105 μ M DMS

Since the treatment of L1210 cells with DMS resulted in a very rapid but surprisingly small increase in ADP-ribosylated protein, this prompted an investigation of fluctuations in NAD⁺ levels in the cell during this time.

Cells were taken at a density of 4×10^5 /ml and DMS added as described in the previous section. During treatment with DMS cells remained impermeable to trypan blue. NAD⁺ levels were then estimated as described in Section 2.2.16 except that during processing cells were not centrifuged since many of the time points chosen for analysis would have been by-passed during centrifugation. Instead all the medium was very carefully aspirated off and then 50% (v/v) EtOH added. A comparison of control cells processed in these two different ways showed essentially the same NAD⁺ levels. The results of this analysis are shown in Fig. 6.3. A drastic drop in NAD⁺ levels to about 11% of control occurred within 2 minutes of addition of DMS. At 5 minutes the NAD⁺ level was still only 12.3% of the control. Thereafter levels began to rise and were approx. 54% of control by 20 minutes. Control levels of NAD⁺ did not vary appreciably during this time (see shaded area of Fig. 6.3). The time course of NAD⁺ drop and its subsequent rise agreed very well

Fig. 6.3 The Effect of 105 μ M DMS on the NAD⁺ Content of L1210 Cells

The NAD⁺ content of control and DMS-treated cells was determined as described in Section 2.2.16 and is represented as the mean percentage (of 3 samples) relative to control. The error is the standard error of the mean (S.E.M.).

Fig. 6.3

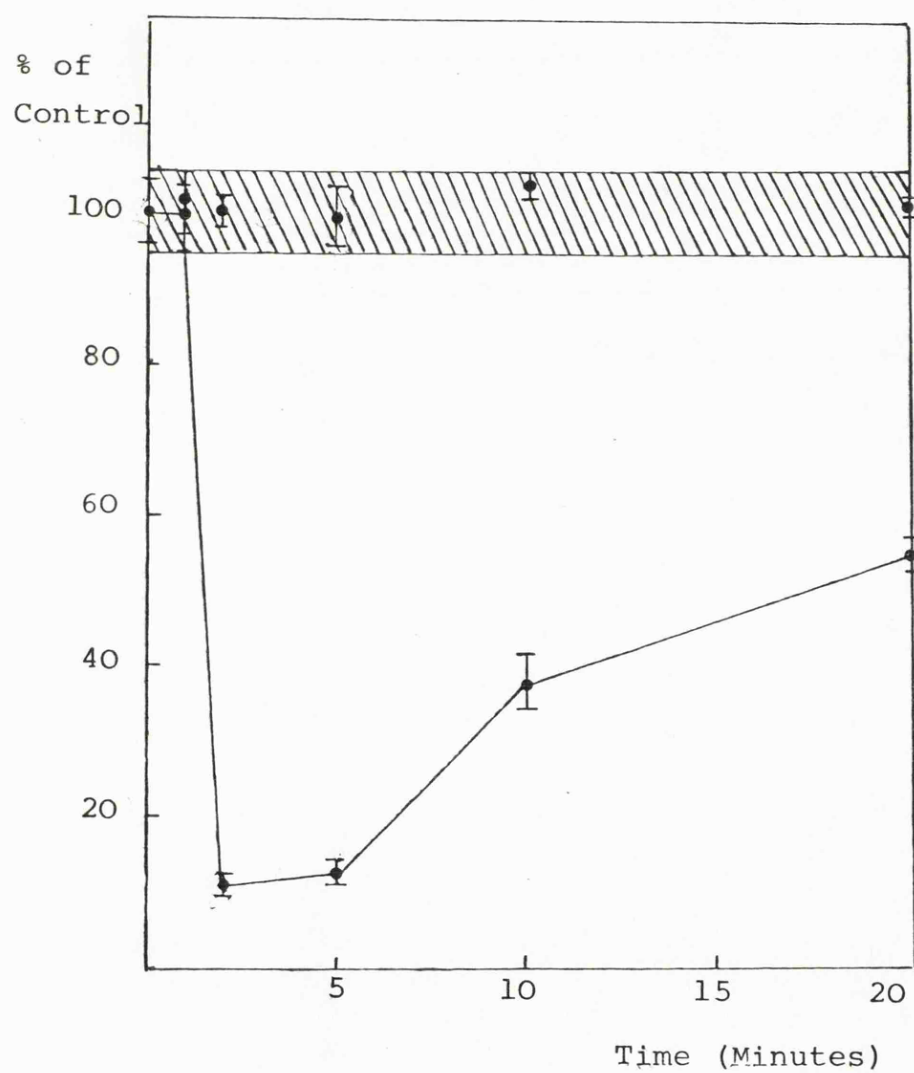


Table 6.3 Effect of Treatment with 105 μ M DMS on the
NAD⁺ Content of Mouse L1210 Cells

Duration of DMS Treatment (Min)	Change in A ₅₅₆ in 5 minutes	pmol NAD ⁺ /10 ⁶ cells	pmol NAD ⁺ /μg DNA	% of Control
0	0.070 \pm 0.003	1060 \pm 45	108.2 \pm 4.6	100.0
1	0.069 \pm 0.003	1050 \pm 46	107.1 \pm 4.7	99.0
2	0.007 \pm 0.001	113 \pm 11	11.5 \pm 1.2	10.7
5	0.009 \pm 0.001	130 \pm 14	13.3 \pm 1.4	12.3
10	0.025 \pm 0.003	373 \pm 45	38.1 \pm 4.6	35.2
20	0.038 \pm 0.002	570 \pm 30	58.2 \pm 3.1	53.8

The change in absorbance at 556nm represented is that of 10⁶ cells. DNA values averaged 9.8 \pm 0.68μg/10⁶ cells. All errors are represented as the standard error of the mean of three samples (S.E.M.).

with the increase and subsequent decrease in ADP-ribosylation observed on caesium chloride density gradient centrifugation. However, the extent of the NAD^+ drop was significantly more than the increase in ADP-ribosylation. Furthermore, the time taken for NAD^+ to reach control levels after the drop appeared to be greater than that observed for ADP-ribosylated protein.

The levels of NAD^+ in the cell observed during treatment with DMS are shown in Table 6.3. Control levels of NAD^+ averaged $1060\text{pmol}/10^6$ cells or $108.2\text{pmol}/\mu\text{g}$ DNA. These levels fell to a minimum of $113\text{pmol}/10^6$ cells or $11.5\text{pmol}/\mu\text{g}$ DNA at 2 minutes after addition of DMS.

6.4 DISCUSSION

Alkylating agents such as dimethyl sulphate are essentially electrophilic (Miller, 1970) and as a result react with the many nucleophilic sites on DNA. Sites on DNA most reactive towards DMS are particularly the N_7 of guanine and to a lesser extent the N_7 of adenine (Lawley, 1966). The introduction of alkyl groups at the N_7 of guanine labilizes the glycosidic linkage between the N_9 of the purine and the C_1 of the deoxyribose and so makes the bond more susceptible to hydrolysis. Although the N_7 of guanine is not involved in hydrogen bonding the alkylation of DNA at this position leads to the production of apurinic sites and subsequent single strand breakage. Alkylation by DMS causes 'small patch' repair with only 3-4 nucleotides being excised by exonucleolytic action

(Regan & Setlow, 1974).

Treatment of mouse L1210 cells with 105 μ M DMS resulted in a rapid increase in ADP-ribosylated protein and a similarly rapid decrease in NAD⁺ levels within 2-5 minutes. The extent of NAD⁺ drop, to 11% of control, far exceeded the apparent increase in ADP-ribosylation of proteins which was only 167% of the control.

Harrap & Furness (1973) have stated that some alkylating agents interfere with the plasma membrane and subsequently make cells 'leaky'. However, cells remained impermeable to trypan blue during treatment with DMS and as such the drop in NAD⁺ cannot be explained by NAD⁺ 'leaking' from the cell. The subsequent increase in NAD⁺ with time supports this contention.

Once the synthesized poly(ADPR) has served its function in the response to DNA damage it is presumably degraded by poly(ADPR) glycohydrolase. Since single-stranded DNA is produced on DNA damage, it is probable that the glycohydrolase will be inhibited by its presence (Hayaishi & Ueda, 1974). Although it is unlikely that the single-stranded breaks in DNA are repaired in a matter of minutes, the results do suggest that some poly(ADPR) glycohydrolase activity exists in the presence of DNA damage i.e. some turnover of poly(ADPR) is apparent. Juarez-Salinas et al. (1979) also proposed that the newly synthesized poly(ADPR) turns over rapidly. Furthermore, the fact that Durkacz et al. (1980) found it necessary to use much higher concentrations of DMS to observe an increase in poly(ADPR) similar to the decrease

in NAD^+ , supports this theory. However, a number of factors remain unknown, namely: (i) the proximity of poly(ADPR) glycohydrolase to the damaged region on DNA, (ii) the particular aspect of single-stranded DNA which causes inhibition of the glycohydrolase, and (iii) the extent to which DNA is alkylated by DMS. Consequently, it is not possible to speculate on an explanation for this observation at present; the process is apparently very complex.

The increase in ADP-ribosylation of protein induced by DMS could only be measured in terms of percentage of radioactivity incorporated whereas the decrease in NAD^+ could be measured with respect to actual levels in the cell. It was conceivable that the apparent differences in the extent of the response might be more similar if estimated in terms of the quantity of material present. In an attempt to clarify the situation, an estimate of the quantity of ADPR on protein before and after treatment with DMS was made with a knowledge of approximate levels of DNA and RNA in the cell and the relative percentage of incorporation of $[^3\text{H}]$ adenosine into these species. The increase in poly(ADPR) on treatment with DMS was estimated to be $0.414\mu\text{g}/10^6$ cells. The decrease in NAD^+ observed was $0.587\mu\text{g}/10^6$ cells. Hence, 70.5% of the drop in NAD^+ could be accounted for by the increase in poly(ADPR). Although this estimation is only approximate and is dependent on a number of assumptions, it is important because it demonstrates that the decrease in NAD^+ and increase in poly(ADP-ribosylation) are of the same order.

The rapidity of the response of the poly(ADPR) system to DMS treatment supports the finding of Berger et al. (1979a) that no synthesis of poly(ADPR) synthetase is necessary during this period. The ability of the cells to recover their NAD^+ levels after a 90% depletion suggested that their metabolism was not drastically altered by the drop. The greater time required for recovery of NAD^+ levels compared to poly(ADPR) levels was probably a result of the time necessary for resynthesis of NAD^+ and indeed its precursors. It appears that both poly(ADPR) synthetase and NAD^+ might be kept in large reserves in 'anticipation' of DNA damage.

Analysis of ADP-ribosylation of protein by CsCl density gradient centrifugation indicated that the chain length of poly(ADPR) synthesized on DMS treatment was greater than that in control cells; the average buoyant density of [^3H]adenosine-labelled protein increased on treatment. This observation was similar to that in stationary cells and is consistent with the finding that it is an increase in poly(ADPR) rather than mono(ADPR) or small chain oligo(ADPR) that occurs in response to DNA damage.

The response of the poly(ADPR) system to DNA damage was rapid and was similar to that observed by Juarez-Salinas et al. (1979) in MNNG-treated SVT2 cells and by Skidmore et al. (1979) and Benjamin & Gill (1980) in γ and x-irradiated cells, respectively. The differences in the response to DNA damage observed in various systems may be dependent on a number of factors, including: (i) the

state of the cell at the time of treatment, (ii) the extent to which the agent may permeate the cell, (iii) the half-life of the damaging agent (the half-life of DMS is approximately 10 minutes), (iv) the sites of attack on DNA by the agent, and (v) the activity of poly(ADPR) glycohydrolase.

The work described in this chapter is a preliminary analysis of the effect of DNA damaging agents on ADP-ribosylation in vivo. Although previous analyses have demonstrated an increase in poly(ADPR) synthetase activity (e.g. Durkacz et al., 1980), the results presented here show for the first time that an increase in protein ADP-ribosylation occurs in response to DNA damage. The isolation method developed during this work will allow both an analysis of the specific protein(s) ADP-ribosylated as a result of DNA damage and the average chain length of ADPR on these proteins. Such determinations are of paramount importance in elucidating the role of ADP-ribosylation in nuclear function.

CHAPTER 7

G E N E R A L D I S C U S S I O N

Although ADP-ribosylation of nuclear proteins in the eukaryotic cell has been studied for a number of years, no clear-cut function has yet been assigned to the modification. Because of the inability of the immediate precursor of ADPR, NAD^+ , to enter the cell, most studies to date have been carried out in vitro using isolated nuclei radiolabelled with NAD^+ . The majority of nuclear isolation procedures employed are likely to alter the structure of chromatin and may even result in fragmentation of DNA. As an integral part of the chromatin the poly(ADPR) system will inevitably be affected by these changes. Clearly, to reveal the true function of ADP-ribosylation efforts must be made to analyse the system in vivo. Since the poly(ADPR) system is involved in the modification of nuclear proteins, then the isolation of proteins ADP-ribosylated in vivo will prove extremely useful in elucidating the role of this modification.

The aim of this thesis was to develop a method for the isolation of proteins ADP-ribosylated in vivo and to subsequently study their characteristics. In order to obtain an overall view of ADP-ribosylation in vivo it was necessary to make a non-selective and therefore total isolation of the proteins ADP-ribosylated. The method developed here allowed such an isolation to be made; it was not dependent on any particular characteristics of either poly(ADPR), mono(ADPR) or their linkage to protein but merely on the different buoyant densities between ADP-ribosylated protein and DNA and RNA on

caesium chloride density gradient centrifugation. Furthermore, since the method did not depend on any special characteristics of the cells used it can be easily adopted for other systems. Indeed, it has already proved useful in the analysis of ADP-ribosylation in rat pancreas (Whish & Poirier, 1980) and germinating wheat embryo (N. Abed, personal communication).

Characterization of the isolated, in vivo ADP-ribosylated protein revealed a number of similarities and differences with that in vitro. The average chain length of 1.3 was short compared with that in vitro but was consistent with values observed in a number of in vivo systems (Ueda et al., 1975; Adamietz et al., 1978; Young & Sweeney, 1978, 1979). The ADPR-protein linkage was sensitive to alkali and neutral hydroxylamine. The extent of these sensitivities, however, differed from that in vitro; the existence of some ADPR-protein linkages resistant to alkali was observed and also relatively few ADPR-protein linkages were sensitive to neutral hydroxylamine. It is plausible that during nuclear isolation the state of the chromatin is altered in such a way as to mimic natural situations. As a result the specific ADP-ribosylation of certain protein fractions may occur. Since ADP-ribosylation in vitro results in a greater degree of poly(ADPR) and also of hydroxylamine-sensitive linkages compared with that in vivo it is conceivable that this ADP-ribosylation is, to some extent, a result of nuclear isolation. Hilz et al., (1980) showed that levels of NH_2OH -sensitive, mono(ADPR)-protein conjugates

corresponded linearly to mean cell doubling time for a large number of tissues, high levels in this subfraction being associated with low proliferation rates and vice versa. Indeed, the relatively short mean generation time in L1210 cells was associated with low levels of hydroxylamine-sensitive conjugates (see Chapter 4).

The effect of inhibitors of poly(ADPR) synthesis on ADP-ribosylation of proteins in vivo revealed that the process is more complex than that in vitro. The results of other workers (e.g. Bredehorst et al., 1980) support this finding. However, further investigations are necessary before a better understanding of these differences can be acquired.

The addition of DMS resulted in a rapid lowering of NAD^+ levels as has been observed in isolated nuclei and permeabilized cells (e.g. Durkacz et al., 1980). In addition, an increase in protein ADP-ribosylation was observed. This increase in ADP-ribosylation appeared to be associated with an increase in average chain length of ADPR bound to protein. A similar increase was observed in stationary phase cells. Increased levels in poly(ADPR) rather than mono(ADPR) appear to be associated with the cessation of DNA synthesis and DNA strand breakage. In fact DNA synthesis is reduced in the presence of DNA damage (Roberts, 1978).

Apparently a number of different ADP-ribosylation processes may exist according to different states or functions of the cell. These processes appear to fall into two different categories: (i) ADP-ribosylation by

mono(ADPR) and perhaps short chain oligo(ADPR), observed under a number of different circumstances and possibly involved in the maintenance of a certain status of chromatin for an extended period of time or even permanently such as in the fully differentiated cell, and (ii) poly(ADP-ribosyl)ation, a substantial increase of which is observed in response to more drastic situations such as DNA damage, the polymer being rapidly synthesized and degraded. The increase in poly(ADP-ribosyl)ation may be necessary for a greater degree of accessibility.

Mono(ADP-ribosyl)ation occurs at low levels compared to the poly(ADP-ribosyl)ation induced by DNA damage and is probably turned over relatively slowly, as is evident from the half-life of ADP-ribosylated protein observed in L1210 cells (see Chapter 4). Since poly(ADPR) glycohydrolase does not cleave mono(ADPR) from protein, this supports the theory that the role of mono(ADP-ribosyl)ation is different from that of poly(ADP-ribosyl)ation.

These differences in ADP-ribosylation suggest that different synthesizing activities may be present. The observation that the substantial increase in poly(ADPR) on DNA damage is caused by a 'latent' poly(ADPR) synthetase activity (Berger et al., 1978a; Durkacz et al., 1980) supports this theory. Since the increase is only transient, then a comparable increase in poly(ADPR) glycohydrolase activity is necessary to cope with the rapid degradation of poly(ADPR) required to permit reversion of the chromatin to its normal status. Indeed, the existence of a 'masked' or latent form of poly(ADPR)

glycohydrolase has been reported by Kidwell et al. (1980). Consequently, during the response to DNA damage there must exist a co-ordinated action of poly(ADPR) synthetase and glycohydrolase activities.

The development of a method for the isolation of total proteins ADP-ribosylated in vivo provides a basis for a whole spectrum of experiments. The great potential of this technique is that it may be adopted for a vast number of analyses in virtually any system because it is non-selective. The extent of ADP-ribosylation, average chain length, linkage of ADPR to protein and proteins modified may all be determined. In fact with the development or modification of a gel electrophoretic system of greater resolution it might become possible to analyse the ADP-ribosylation of a single protein in detail. 2D gel electrophoresis in combination with autoradiography may prove particularly useful in this respect. With the development of this technique and the vast number of analyses potentially dependent on it, we may be on the threshold of discovering the role of ADP-ribosylation in nuclear function.

REFERENCES

- Adamietz, P., Bredehorst, R., Oldekop, M. & Hilz, H. (1974) FEBS Lett. 43, 318-322
- Adamietz, P., Oldekop, M. & Hilz, H. (1975) J. Biochem. 77, 4p
- Adamietz, P. & Hilz, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 527-534
- Adamietz, P., Bredehorst, R. & Hilz, H. (1978a) Biochem. Biophys. Res. Commun. 81, 1377-1383
- Adamietz, P., Bredehorst, R., & Hilz, H. (1978b) Eur. J. Biochem. 91, 317-326
- Adamietz, P., Klapproth, K. & Hilz, H. (1979) Biochem. Biophys. Res. Commun. 91, 1232-1238
- Adamietz, P., Braeuer, H. & Hilz, H. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Aloyo, V.J. (1979) Anal. Biochem. 99, 161-164
- Ballal, N.R. & Busch, H. (1973) Cancer Res. 33, 2737-2743
- Ballal, N.R., Goldknopf, I.L., Goldberg, P.A. & Busch, H. (1974) Life Sci. 14, 1835-1845
- Benjamin, R.C. & Gill, D.M. (1978) J. Supramol. Struct. Suppl. 2, 74.
- Benjamin, R.C. & Gill, D.M. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Berger, N.A. & Johnson, E.S. (1976) Biochim. Biophys. Acta 425, 1-17
- Berger, N.A., Petzold, S.J. & Johnson, E.S. (1977) Biochim. Biophys. Acta 478, 44-58
- Berger, N.A. & Weber, G. (1977) J. Natl. Cancer Inst. 58, 1167-1169
- Berger, N.A., Weber, G. & Kaichi, A.S. (1978a) Biochim. Biophys. Acta 519, 87-104
- Berger, N.A., Weber, G., Kaichi, A.S. & Petzold, S.J. (1978b) Biochim. Biophys. Acta 519, 105-117
- Berger, N.A., Adams, J.W., Sikorski, G.W., Petzold, S.J., Shearer, W.T. (1978c) J. Clin. Invest. 62, 111-118

- Berger, N.A., Kaichi, A.S., Steward, P.G., Klevecz, R.R., Forrest, G.L. & Gross, S.D. (1978d) Exp. Cell Res. 117, 127-135
- Berger, N.A., Sikorski, G.W., Petzold, S.J. & Kurohara, K.K. (1979a) J. Clin. Invest. 63, 1164-1171
- Berger, N.A., Petzold, S.J. & Berger, S.J. (1979b) Biochim. Biophys. Acta 564, 90-104
- Berger, N.A. & Sikorski, G.W. (1980a) Biochem. Biophys. Res. Commun. 95, 67-72
- Berger, N.A. & Sikorski, G.W. (1980b) Proceedings of NATO Meeting on Chromosome Damage and Repair. In the press
- Berger, N.A., Sikorski, G.W., Petzold, S.J. & Kurohara, K.K. (1980a) Biochemistry 19, 289-293
- Berger, N.A., Sikorski, G.W. & Petzold, S.J. (1980b) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Birnie, G.D. (1978) In: Centrifugal Separations in Molecular and Cell Biology (Birnie, G.D. & Rickwood, D., eds.) 169-217, Butterworths, London
- Bjursell, G. & Reichard, P. (1973) J. Biol. Chem. 248, 3904-3909
- Bradford, M.M. (1976) Anal. Biochem. 72, 248-254
- Bredehorst, R., Ferro, A.M. & Hilz, H. (1978a) Eur. J. Biochem. 82, 105-113
- Bredehorst, R., Ferro, A.M. & Hilz, H. (1978b) Eur. J. Biochem. 82, 115-121
- Bredehorst, R., Wielckens, K., Gartemann, A., Lengyel, H., Klapproth, K. & Hilz, H. (1978c) Eur. J. Biochem. 92, 129-135
- Bredehorst, R., Goebel, M., Renzi, F., Kittler, M., Klapproth, K. & Hilz, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1737 - 1743
- Bredehorst, R., Lengyel, H. & Hilz, H. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 559-562
- Brightwell, M.D. & Shall, S. (1971) Biochem. J. 125, 67p
- Brightwell, M.D., Leech, C.E., O'Farrell, M.K., Whish, W.J.D. & Shall, S. (1975) Biochem. J. 147, 119-129
- Burzio, L.O. & Koide, S.S. (1970) Biochem. Biophys. Res. Commun. 40, 1013-1020

- Burzio, L.O., Riquelme, P.T., Ohtsuka, E. & Koide, S.S. (1970) Arch. Biochem. Biophys. 173, 306 - 319
- Burzio, L.O. & Koide, S.S. (1971) Biochem. Biophys. Res. Commun. 42, 1185-1190
- Burzio, L.O. & Koide, S.S. (1972) FEBS Lett. 20, 29-32
- Burzio, L.O. & Koide, S.S. (1973) Biochem. Biophys. Res. Commun. 53, 572-579
- Burzio, L.O., Reich, L. & Koide, S.S. (1975) Proc. Soc. Exp. Biol. Med. (N.Y.) 149, 933-938
- Burzio, L.O. & Koide, S.S. (1977) Ann. N.Y. Acad. Sci. 286, 398-407
- Burzio, L.O., Koide, S.S., Domenech, P.P. & Ruiz-Carrillo, A. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Butt, T.R., Brothers, J.F., Giri, G.P. & Smulson, M.E. (1978) Nucleic Acids Res. 5, 2775-2788
- Butt, T.R., Jump, D.B. & Smulson, M.E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1628-1632
- Byrne, R.H., Stone, P.R. & Kidwell, W.R. (1978) Exp. Cell Res. 115, 277-283
- Caplan, A.I. & Rosenberg, M.J. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1852-1857
- Caplan, A.I., Niedergang, C., Okazaki, H. & Mandel, P. (1979a) Arch. Biochem. Biophys. 198, 60-69
- Caplan, A.I., Niedergang, C., Okazaki, H. & Mandel, P. (1979b) Dev. Biol. 72, 102-109
- Caplan, A.I., Niedergang, C., Okazaki, H. & Mandel, P. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Chambon, P., Weill, J.D. & Mandel, P. (1963) Biochem. Biophys. Res. Commun. 11, 39-43
- Chambon, P., Weill, J.D., Doly, J., Strosser, M.T. & Mandel, P. (1966) Biochem. Biophys. Res. Commun. 25, 638-643
- Clark, J.B., Ferris, G.M. & Pinder, S. (1971) Biochim. Biophys. Acta 238, 82-85
- Claycomb, W.C. (1976) Biochem. J. 154, 387-393
- Cleaver, J.E. & Thomas, G.H. (1969) Biochem. Biophys. Res. Commun. 36, 203-208

Colyer, R.A., Burdette, K.E. & Kidwell, W.R. (1973) Biochem. Biophys. Res. Commun. 53, 960-965

Davies, M.I., Halldorsson, H., Shall, S. & Skidmore, C.J. (1976) Biochem. Soc. Transac. 4, 635-637

Davies, M.I., Shall, S. & Skidmore, C.J. (1977) Biochem. Soc. Transac. 5, 949-950

Delange, R.J. & Smith, E.L. (1971) Ann. Rev. Biochem. 40, 279-314

Dick, C. & Johns, E.W. (1968) Exp. Cell Res. 51, 626-632

Dietrich, L.S., Jaus, H. & Siebert, G. (1973) FEBS Lett. 37, 228-230

Dietrich, L.S. & Siebert, G. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1133 - 1140

Dietrich, L.S. & Siebert, G. (1974) Biochem. Biophys. Res. Commun. 56, 283-289

Doly, J. & Petek, F. (1966) C.R. Acad. Sci. (Paris) 263, 1341-1344

Doly, J. & Mandel, P. (1967) C.R. Acad. Sci. (Paris) 264, 2687-2690

Durkacz, B.W., Omidiji, O., Gray, D.A. & Shall, S. (1980) Nature 283, 593-596

Fahrney, D.E. & Gold, A.M. (1963) J.A.C.S. 85, 997-1000

Farzaneh, F. & Pearson, C.K. (1978) Biochem. Biophys. Res. Commun. 84, 537-543

Farzaneh, F. & Pearson, C.K. (1979) Dev. Biol. 72, 254-265

Farzaneh, F., Zalin, R.J. & Shall, S. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.).

Ferro, A.M., Minaga, T., Piper, W.N. & Kun, E. (1978) Biochim. Biophys. Acta 519, 291-305

Ferro, A.M. & Oppenheimer, N.J. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 809-813

Finch, J.T. & Klug, A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1897-1901

- Fox, T.O. & Pardee, A.B. (1970) Science 167, 80-82
- Fugimura, S. & Sugimura, T. (1971) In: Methods in Enzymol. (McCormick, D.B. & Weight, L.D. eds.) 18B, 223-230
- Furneaux, H.M. & Pearson, C.K. (1978a) Biochem. Soc. Transac. 6, 753-755
- Furneaux, H.M. & Pearson, C.K. (1978b) Biochem. Soc. Transac 7, 697-699
- Furneaux, H.M. & Pearson, C.K. (1980) Biochem. J. 187, 91-103
- Futai, M., Mizuno, D. & Sugimura, T. (1968) J. Biol. Chem. 243, 6325-6329
- Gautschi, J.R., Kern, R. M. & Painter, R.B. J. Mol. Biol. 80, 393-403
- Ghani, Q.P. & Hollenberg, M. (1978a) Biochem. J. 170, 387-394
- Ghani, Q.P. & Hollenberg, M. (1978b) Biochem. Biophys. Res. Commun. 81, 886-891
- Gill, D.M. (1972) J. Biol. Chem. 247, 5964-5971
- Gill, D.M., Caplan, R.A., Bredlau, C.E. & Meren, R. (1974) Fogarty International Center Proceedings 26, 85-99 (Harris, M., ed.)
- Giri, C.P., West, M.H.P. & Smulson, M.E. (1978a) Biochemistry 17, 3495-3500
- Giri, C.P., West, M.H.P., Ramirez, M.L. & Smulson, M.E. (1978b) Biochemistry 17, 3501-3504
- Goebel, M., Stone, P.R., Lengyel, H. & Hilz, H. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 13-21
- Goodwin, G.H. & Johns, E.W. (1977) In: Methods in Cell Biol. 16, 257-267
- Gottesfeld, J.M., Garrand, W.T., Bagi, G., Wilson, R.F. & Bonner, J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2193-2197
- Gottesfeld, J.M., Murphy, R.F. & Bonner, J. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4404-4408
- Halldorsson, H., Gray, D.A. & Shall, S. (1978) FEBS Lett. 85, 349-352
- Haines, M.E., Johnston, I.R., Mathias, A.P. & Ridge, D. (1969) Biochem. J. 115, 881-887

- Harrap, K.P. & Furness, M.E. (1973) Eur. J. Cancer 9, 343-351
- Hasegawa, S., Fujimura, S., Shimizu, Y. & Sugimura, T. (1967) Biochim. Biophys. Acta. 149, 369-376
- Hashida, T., Ohgushi, H. & Yoshihara, K. (1979) Biochem. Biophys. Res. Commun. 88, 305-311
- Hayaishi, O. & Ueda, K. (1974) Fogarty International Center Proceedings 26, 69-76 (Harris, M., ed.)
- Hayaishi, O. & Ueda K. (1977) Ann. Rev. Biochem. 46, 95-116
- Hayaishi, O. & Ueda, K., Kawaichi, M., Ogata, N., Ikai, K., Oka, J., Kim, H.R., Ito, S., Shizuta, Y. & Okayama, H. (1979) Fed. Proc. Fed. Am. Soc. Exp. Biol. 38, 618
- Hillyard, D., Rechsteiner, M. & Olivera, B. (1973) J. Cell Physiol. 82, 165-179
- Hilz, H., Hlavica, P. & Betram, B. (1963) Biochem. Z. 338, 283-299
- Hilz, H. & Kittler, M. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 1793-1796
- Hilz, H. & Kittler, M. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1693-1704
- Hilz, H., Adamietz, P., Bredehorst, R. & Lieber, U. (1974) Fogarty International Center Proceedings 26, 47-67 (Harris, M., ed.)
- Hilz, H., Wiegers, U. & Adamietz, P. (1975) Eur. J. Biochem. 56, 103-108
- Hilz, H. & Stone, P.R. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1-58
- Hilz, H., Bredehorst, R., Wielckens, K., Gartemann, A., & Adamietz, P. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Hollenberg, M. (1971) Circ. Res. 28, 148-157
- Hogeboom, G. & Schneider, W. (1952) J. Biol. Chem. 197, 611-620
- Hotlund, J., Kristensen, T. & Sletten, K. (1980) Biochem. J. 185, 779-782
- Huberman, J.A. & Riggs, A.D., (1968) J. Mol. Biol. 32, 327-341
- Hutchinson, D.J., Ittensoln, O.L. & Bjerrgaard, M.R. (1966) Exp. Cell Res. 42, 157-170

- Ikai, K., Ueda, K., Fukushima, M., Nakamura, T. & Hayaishi, O. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3682-3685
- Inagaki, F., Miyazawa, T., Miwa, M., Saito, H. & Sugimura, T. (1978) Biochem. Biophys. Res. Commun. 85, 415-420
- Ito, S., Shizuta, Y. & Hayaishi, O. (1979) J. Biol. Chem. 254, 3647-3651
- Jacobson, E.L. & Narasimhan, G. (1979) Fed. Proc. Fed. Am. Soc. Exp. Biol. 38, 619
- Jacobson, M.K. & Jacobson, E.L. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. 37, 1347
- Jacobson, M.K., Sims, J.L., Juarez-Salinas, H., Levi, V., Barton, R.A. & Jacobson, E.L. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Janakadevi, K. (1978) Exp. Cell Res. 112, 345-351
- Janakadevi, K. & Koh, Ch. (1974) Biochemistry 13, 1327-1330
- Janakadevi, K. & States, J.C. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Juarez-Salinas, H., Sims, J.L. & Jacobson, M.K. (1979) Nature 282, 740-741
- Jump, D.B., Butt, T.R. & Smulson, M.E. (1979) Biochemistry 18, 983-990
- Jump, D.B. & Smulson, M. (1980) Biochemistry 19, 1024-1030
- Jump, D.B., Butt, T.R. & Smulson, M.E. (1980a) Biochemistry 19, 1031-1037
- Jump, D.B., Sudhakar, S. and Smulson, M.E. (1980b) Chem. Biol. Interactions. In the press
- Kanai, Y., Sugimura, T., Aosuka, S-I. & Yokohari, R. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Kawaichi, M., Ueda, K. & Hayaishi, O. (1980) J. Biol. Chem. 255, 816-819
- Khan, M.G. & Shall, S. (1976) Biochem. Soc. Transac. 4, 778-781

Kidwell, W.R. & Burdette, K.E. (1974) Biochem. Biophys. Res. Commun. 61, 766-773

Kidwell, W.R. & Mage, M.G. (1976) Biochemistry 15, 1213-1217

Kidwell, W.R., Store, P.R. & Johnson, G.S. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. and Sugimura, T., eds.)

Kitamura, A., Tanigawa, Y., Yamamoto, T., Kawamura, M., Doi, S. & Shimoyama, Y. (1979) Biochem. Biophys. Res. Commun. 87, 725-733

Kleinsmith, L.J. (1978) In: The Cell Nucleus (Busch, H., ed.) 6, 221-261. Academic Press

Kristensen, T. & Hotlund, J. (1976) Eur. J. Biochem. 70, 441-446

Kristensen, T. & Hotlund, J. (1978) Eur. J. Biochem. 88, 495-501

Kristensen, T. & Hotlund, J. (1980) J. Chromatogr. 192, 494-499

Lawley, P.D. (1966) Prog. Nucl. Acid Res. Mol. Biol. 5, 89-131

Lehmann, A.R. & Kirk-Bell, S. (1972) Eur. J. Biochem. 31, 438-445

Lehmann, A.R. & Shall, S. (1972) FEBS Lett. 26, 181-184

Lehmann, A.R., Kirk-Bell, S., Shall, S. & Whish, W.J.D. (1974) Exp. Cell Res. 83, 63-72

Leiber, U., Kittler, M.K. & Hilz, H. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1347-1350

Levi, V., Jacobson, E.L. & Jacobson, M.K. (1978) FEBS Lett. 88, 144-146

Lichtenwalner, D.M. & Suhadolnik, R.J. (1979) Biochemistry 18, 3749-3755

Lorimer, W.S., Stone, P.R. & Kidwell, W.R. (1977) Exp. Cell Res. 106, 261-266

Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T. & Futai, M. (1970) J. Biol. Chem. 245, 4317-4320

Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T., Sugimura, T. & Futai, M. (1970) J. Biol. Chem. 254, 3606-3611

- McLachlan, J., Bateman, M. & Wolpert, L. (1976) *Nature*, 264, 267-269
- Meuth, M., Anfreite, E. & Reichard, P. (1976) *Eur. J. Biochem.* 71, 39-43
- Miller, E.G. (1975a) *Biochim. Biophys. Acta* 395, 191-200
- Miller, E.G. (1975b) *Biochem. Biophys. Res. Commun.* 65, 280-286
- Miller, J.A. (1970) *Cancer Res.* 30, 559-576
- Minaga, T., Morton, L.J., Piper, W.N. & Kun, E. (1978) *Eur. J. Biochem.* 91, 577-585
- Minaga, T., Romaschin, A.D., Kirsten, E. & Kun, E. (1979) *J. Biol. Chem.* 254, 9663-9668
- Miwa, M. & Sugimura, T. (1971) *J. Biol. Chem.* 246, 6362-6364
- Miwa, M., Tanaka, M., Matsushima, T. & Sugimura, T. (1974) *J. Biol. Chem.* 249, 3475-3482
- Miwa, M., Nakatsugawa, K., Hara, K., Matsushima, T. & Sugimura, T. (1975) *Arch. Biochem. Biophys.* 167, 54-60
- Miwa, M., Oda, K., Segawa, K., Tanaka, M., Sakura, R., Matsushima, T. & Sugimura, T. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* 357, 7-8p
- Miwa, M., Saito, H., Sakura, H., Saikawa, N., Watanabe, F., Matsushima, T., & Sugimura, T. (1977) *Nucleic Acids Res.* 4, 3997-4005
- Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S. & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 595-599
- Miyakawa, N., Ueda, K., Hayaishi, O. (1972) *Biochem. Biophys. Res. Commun.* 49, 239-245
- Moroika, K., Tanaka, K., Nokuu, T., Ishizawa, M. & Ono, T. (1979) *Gann* 70, 37-46
- Morton, R.K. (1958) *Nature*, 181, 540-542
- Morton, R.K. (1961) *Australian J. Sci.* 24, 260-278
- Müller, W.E.G., Totsuka, A., Nusser, I., Obermeier, J., Rhode, H. & Zahn, R.K. (1974) *Nucleic Acids Res.* 1, 1317-1327
- Müller, W.E.G., Rohde, H.J., Steffen, R., Maidhof, A., Lachmann, M., Zahn, R.K. & Umezawa, H. (1975) *Cancer Res.* 35, 3673-3681

- Müller, W.E.G. & Zahn, R.K. (1976) *Experientia* 31, 1014-1015
- Mullins, D.W., Giri, C.P. & Smulson, M.E. (1977) *Biochemistry* 16, 506-513
- Nagao, M., Yamada, M., Miwa, M. & Sugimura, T. (1972) *Biochem. Biophys. Res. Commun.* 48, 219-225
- Nduka, N., Skidmore, C.J. & Shall, S. (1980) *Eur. J. Biochem.* 105, 525-530
- Niedergang, C., Okazaki, H. & Mandel, P. (1978) *Anal. Biochem.* 88, 20-28
- Niedergang, C., Okazaki, H. & Mandel, P. (1979) *Eur. J. Biochem.* 102, 43-57
- Nishizuka, Y., Ueda, K., Nakazawa, K., & Hayaishi, O. (1967) *J. Biol. Chem.* 242, 3164-3171
- Nishizuka, Y., Ueda, K., Honjo, T. & Hayaishi, O. (1968) *J. Biol. Chem.* 243, 3765-3767
- Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M. & Hayaishi, O. (1969) *Cold Spring Harbor Symp. Quant. Biol.* 34, 781-786
- Nisselbaum, J.S. & Green, S. (1969) *Anal. Biochem.* 27, 212-217
- Nolde, S. & Hilz, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* 353, 505-513
- Ogata, N., Ueda, K. & Hayaishi, O. (1980) *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins*, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Ohtsu, E. & Nishizuka, Y. (1971) *Methods in Enzymol.* 18B, 127-131
- Okayama, H., Edson, D.M., Fukushima, M., Ueda, K. & Hayaishi, O. (1977) *J. Biol. Chem.* 252, 7000-7005
- Okayama, H. & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun.* 84, 755-762
- Okayama, H., Ueda, K. & Hayaishi, O. (1978a) *Proc. Natl. Acad. Sci. U.S.A.* 75, 1111-1115
- Okayama, H., Honda, M. & Hayaishi, O. (1978b) *Proc. Natl. Acad. Sci. U.S.A.* 75, 2254-2257
- Okazaki, H., Niedergang, C. & Mandel, P. (1976) *FEBS Lett.* 62, 255-258

Okazaki, H., Niedergang, C. & Mandel, P. (1980a)
Biochimie. In the press

Okazaki, H., Niedergang, C., Couppez, M., Martinage, A.,
Sautierre, P. & Mandel, P. (1980b) FEBS Lett. 110, 227-
229

Okolie, E.E. & Shall, S. (1979) Clin. Exp. Immunol. 36,
151-164

Olivera, B.M., Rechsteiner, M.C., Ramos, P.M., Cruz, L.J.
& Hillyard, D. (1980) Novel ADP-Ribosylations of
Regulatory Enzymes and Proteins, Elsevier, North-Holland,
Inc. (Smulson, M.E. & Sugimura, T., eds.)

Ord, M.G. & Stocken, L.A. (1977) Biochem. J. 161, 583-592

Otake, H., Miwa, M., Fujimura, S. & Sugimura, T. (1969)
J. Biochem. 65, 145-146

Paik, W.R. & Kim, S. (1971) Science, 174, 114-119

Perella, F.W. & Lea, M.A. (1978) Biochem. Biophys. Res.
Commun. 82, 575-581

Pitt-Rivers, R. & Impiombato, F.S.A. (1968) Biochem. J.
109, 825-830

Poirier, G.G. & Savard, P. (1980) Canad. J. Biochem. 58,
509-515

Porteus, J.W., Furneaux, H.M., Pearson, C.K., Lake, C.M.
& Morrison, A. (1979) Biochem. J. 180, 455-463

Preiss, J., Schlaeger, R. & Hilz, H. (1971) FEBS Lett.,
19, 244-246

Purnell, M.R. & Whish, W.J.D. (1977) 11th FEBS Meeting
Abstract, Academic Press, London

Purnell, M.R. & Whish, W.J.D. (1980a) Biochem. Soc.
Transac. 8, 175-176

Purnell, M.R. & Whish, W.J.D. (1980b) Biochem. J. 185,
775-777

Purnell, M.R., Stone, P.R., Surowy, C.S. & Whish, W.J.D.
(1981) Biochem. Soc. Transac. In the press

Randerath, K. & Randerath, E. (1965a) Anal. Biochem. 13
575-579

Randerath, E. & Randerath, K. (1965b) Anal. Biochem. 12,
83-93

- Randerath, K. & Randerath, E. (1967) In: Methods in Enzymol. (Colowick, S.P. & Kaplan, N.O., eds.) 12A, 323-347, Academic Press, New York
- Rastl, E. & Swetly, P. (1978) J. Biol. Chem. 253, 4333-4340
- Razzell, W.E. & Khorana, H.G. (1959) J. Biol. Chem. 234, 2105-2113
- Rechsteiner, M., Hillyard, D. & Olivera, B.M. (1970) Nature, 259, 695-696
- Rechsteiner, M., Catanzarite, V. (1974) J. Cell Physiol. 84, 409-422
- Reeder, R.H., Ueda, K., Honjo, T., Nishizuka, Y. & Hayaishi, O. (1967) J. Biol. Chem. 242, 3172-3179
- Regan, J.O. & Setlow, R.B. (1974) Cancer Res. 34, 3318-3325
- Revel, M. & Mandel, P. (1962) Cancer Res. 22, 456-
- Reynolds, J.A. & Tanford, C. (1970a) J. Biol. Chem. 245, 5161-5165
- Reynolds, J.A. & Tanford, C. (1970b) Proc. Natl. Acad. Sci. U.S.A. 66, 1002-1003
- Rickwood, D., MacGillivray, A.J. & Whish, W.J.D. (1977) Eur. J. Biochem. 79, 589-598
- Riquelme, P.T., Burzio, L.O. & Koide, S.S. (1979a) J. Biol. Chem. 254, 3018-3028
- Riquelme, P.T., Burzio, L.O. & Koide, S.S. (1979b) J. Biol. Chem. 254, 3029-3037
- Roberts, J.H., Stark, P. & Smulson, M.E. (1973) Biochem. Biophys. Res. Commun. 52, 43-50
- Roberts, J.H., Stark, P., Gazzoli, M. & Smulson, M.E. (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. 33, 1491
- Roberts, J.H., Stark, P., Giri, C.P. & Smulson, M.E. (1975) Arch. Biochem. Biophys. 171, 305-315
- Roberts, J.J. (1978) Adv. Radiation Biol. 7, 211-436
- Roitt, I.M. (1956) Biochem. J. 63, 300-307
- Rubin, C.S. & Rosen, O.M. (1975) Ann. Rev. Biochem. 44, 831-887
- Sakura, H., Miwa, M., Tanaka, M., Kanai, Y., Shimada, T., Matsushima, T. & Sugimura, T. (1977) Nucleic Acids Res. 4, 2903-2915
- Sakura, H., Miwa, M., Kanai, Y., Matsushima, T. & Sugimura, T. (1978) Nucleic Acids Res. 5, 4025-4038

- Schüssler, H. (1968) Biochim. Biophys. Acta 151, 383-393
- Schwartz, P.M. & Drach, J.C. (1975) J. Chromatogr. 106, 200-203
- Setaro, F. & Morley, C.D.G. (1977) Anal. Biochem. 81, 467-471
- Shall, S. (1975) J. Biochem. 77, 2p
- Shall, S., Goodwin, P., Halldorsson, H., Khan, G., Skidmore, C. & Tsopanakis, C. (1977) Biochem. Soc. Symp. 42, 103-116
- Shall, S., Nduka, N., Omidiji, O. & Zia'ee, A.A. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Shapiro, A.L., Vinuela, E. & Maizel, J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815-820
- Shima, T., Fujimura, S., Hasegawa, S., Shimizu, Y. & Sugimura, T. (1970) J. Biol. Chem. 245, 1327-1330
- Sims, J.L., Juarez-Salinas, H. & Jacobson, M.K. (1979) Fed. Proc. Fed. Am. Soc. Exp. Biol. 38, 493
- Skidmore, C.J., Davies, M.I., Goodwin, P.M., Halldorsson, H., Lewis, P.J., Shall, S. & Zia'ee, A. (1979) Eur. J. Biochem. 101, 135-142
- Smith, J.A. & Stocken, L.A. (1973) Biochem. Biophys. Res. Commun. 54, 297-300
- Smith, J.A. & Stocken, L.A. (1975) Biochem. J. 147, 523-529
- Smulson, M.E., Stark, P., Gazzoli, M. & Roberts, J. (1975) Exp. Cell Res. 90, 175-182
- Smulson, M.E., Schein, P., Mullins, D.W. & Sudhakar, S. (1977) Cancer Res. 37, 3006-3012
- Smulson, M., Butt, T., Jump, D., Nolan, N., DeCoste, B. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
- Stone, P.R. & Shall, S. (1973) Eur. J. Biochem. 38, 146-152
- Stone, P.R., Whish, W.J.D. & Shall, S. (1973) FEBS Lett. 36, 334-337
- Stone, P. & Hilz, H. (1975) FEBS Lett. 57, 209-212
- Stone, P. & Shall, S. (1975) Exp. Cell Res. 91, 95-100
- Stone, P.R., Bredehorst, R., Kittler, M., Lengyel, H. & Hilz, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 51-56

- Stone, P.R., Lorimer, W.S. & Kidwell, W.R. (1977) Eur. J. Biochem. 81, 9-18
- Stone, P.R., Lorimer, W.S., Ranchalis, J., Danley, M., Kidwell, W.R. (1978) Nucleic Acids Res. 5, 173-184
- Stone, P.R. & Kidwell, W.R. (1980) Biochem. Biophys. Res. Commun. 92, 716-724
- Stratling, W.H., Muller, K. & Zentgraf, H. (1978) Exp. Cell Res. 117, 301-311
- Sudhakar, S., Tew, K.D. & Smulson, M.E. (1979a) Cancer Res. 39, 1405-1410
- Sudhakar, S., Tew, K.D., Schein, P.S., Woolley, P.V. & Smulson, M.E. (1979b) Cancer Res. 39, 1411-1415
- Sugimura, T., Fugimura, S., Hasegawa, S. & Kawamura, Y. (1967) Biochim. Biophys. Acta 138, 438-441
- Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H. & Nagao, M. (1971) Arch. Biochem. Biophys. 147, 660-665
- Sugimura, T., Yamada, M., Miwa, M., Matsushima, T., Hidaka, T., Nagao, M., Inui, N. & Takayama, S. (1973) Biochem. Soc. Transac. 1 642-644
- Suhadolnik, R.J., Baur, R., Lichtenwalner, D.M., Uematsu, T., Roberts, J.H., Sudhakar, S.H. & Smulson, M.E. (1977) J. Biol. Chem. 252, 4134-4144
- Tanaka, M., Miwa, M., Matsushima, T., Sugimura, T. & Shall, S. (1976) Arch. Biochem. Biophys. 172, 224-229
- Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T. & Sugimura, T. (1978) Nucleic Acids Res. 5, 3183-3194
- Tanigawa, Y., Kawamura, M. & Shimoyama, M. (1977) Biochem. Biophys. Res. Commun. 76, 406-412
- Tanigawa, Y., Kawamura, M., Kitamura, A. & Shimoyama, M. (1978a) Biochem. Biophys. Res. Commun. 81, 1278-1285
- Tanigawa, Y., Kitamura, A. & Shimoyama, M. (1978b) Eur. J. Biochem. 92, 261-269
- Tanigawa, Y., Kawakami, K., Inai, Y. & Shimoyama, M. (1980) Biochim. Biophys. Acta 608, 82-95
- Tanuma, S., Enomoto, T. & Yamada, M. (1977) Biochem. Biophys. Res. Commun. 74, 599-605
- Tanuma, S., Enomoto, T. & Yamada, M. (1978) Exp. Cell Res. 117, 421-430

Tanuma, S., Enomoto, T. & Yamada, M. (1979) J. Biol. Chem. 254, 4960-4962

Terada, M., Fujiki, H., Marks, P.A. & Sugimura, T. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6411-6414

Terasima, T. & Tolmach, L.J. (1963) Exp. Cell Res. 30 344-

Teng, C.S., Gallagher, K. & Teng, C.T. (1978) Biochem. J. 176, 1003-1006

Thoma, F. & Koller, Th. (1977) Cell 12, 101-107

Tsopanakis, C., MacLaren, E.A. & Shall, S. (1976) Biochem. Soc. Transac. 4, 774-777

Tsopanakis, C., MacLaren, E.A., Tsopanakis, A. & Shall, S. (1978a) Eur. J. Biochem. 90, 337-345

Tsopanakis, C., Leer, J.C., Nielsen, O.F., Gocke, E., Shall, S. & Westergaard, O. (1978b) FEBS Lett. 93, 297-300

Ueda, K., Reeder, R.H., Honjo, T., Nishizuka, Y. & Hayaishi, O. (1968) Biochem. Biophys. Res. Commun. 34, 379-385

Ueda, K., Oka, J., Narumiya, S., Miyakawa, N. & Hayaishi, O. (1972) Biochem. Biophys. Res. Commun. 46, 516-523

Ueda, K., Okayama, H., Fukushima, M. & Hayaishi, O. (1975a) J. Biochem. 77, 1p

Ueda, K., Omachi, A., Kawaichi, M. & Hayaishi, O. (1975b) Proc. Natl. Acad. Sci. U.S.A. 72, 205-209

Ueda, K., Kawaichi, M., Okayama, H. & Hayaishi, O. (1979) J. Biol. Chem. 254, 679-687

Ueda, K., Kawaichi, M., Oka, J. & Hayaishi, O. (1980) Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier, North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)

Weber, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

Whish, W.J.D., Davies, M.I. & Shall, S. (1975) Biochem. Biophys. Res. Commun. 65, 722-730

Whish, W.J.D. & Poirier, G.G. (1980) Biochem. Soc. Transac. 8, 192

Whitby, A.J., Stone, P.R. & Whish, W.J.D. (1979) Biochem. Biophys. Res. Commun. 90, 1295-1304

Whitby, A.J. (1980) Ph.D. Thesis. University of Bath, England.

Wielckens, K., Sachsenmaier, W. & Hilz, H. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 39-43

Wielckens, K., Garbrecht, M., Kittler, M. & Hilz, H. (1980) Eur. J. Biochem. 104, 279-287

Wong, N.C., Poirier, G.G. & Dixon, G.H. (1977) Eur. J. Biochem. 77, 11-21

Yamada, M. & Sugimura, T. (1973) Biochemistry 12, 3303-3308

Yamada, M., Shimada, T., Nakayasu, M., Okada, H. & Sugimura, T. (1978) Biochem. Biophys. Res. Commun. 83, 1325-1332

Yamamoto, H. & Okamoto, H. (1980) Biochem. Biophys. Res. Commun. 95, 474-481

Yoshihara, K. (1972) Biochem. Biophys. Res. Commun. 47, 119-125

Yoshihara, K. & Koide, S.S. (1973) FEBS Lett. 30, 261-264

Yoshihara, K., Tanigawa, Y. & Koide, S.S. (1974) Biochem. Biophys. Res. Commun. 59, 658-665

Yoshihara, K., Tanigawa, Y., Burzio, L. & Koide, S.S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 289-293

Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. & Ohgushi, H. (1977) Biochem. Biophys. Res. Commun. 78, 1281-1288

Yoshihara, K., Hashida, T., Tanaka, Y., Ohgushi, H., Yoshihara, H. & Kamiya, T. (1978) J. Biol. Chem. 253, 6459-6466

Yoshihara, K., Tanaka, Y., Yoshihara, H., Hashida, T., Ohgushi, H., Arai, R. & Kamiya, T. (1980) In: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)

Young, R.J. & Sweeney, K. (1978) Biochemistry 17, 1901-1908

Young, R.J. & Sweeney, K. (1979) J. Embryol. Exp. Morph. 49, 139-152

Yuki, H., Sempuku, C., Park, M. & Takiura, K. (1972) Anal. Biochem. 46, 123-128

Yukioka, M., Oka, Y., Hasuma, T. & Inoue, A. (1978) FEBS Lett. 86, 85-88

Zatman, L.J., Kaplan, N.O. & Colowick, S.P. (1953) J.Biol. Chem. 200, 197-212

Zlatanova, J.S. & Swetly, P. (1980) Biochem. Biophys. Res. Commun. 92, 1110-1116

PUBLICATIONS

1. Surowy, C.S. & Whish, W.J.D. (1979) 5th EMBO Annual Symposium on Protein-Nucleic Acid Interaction, Abstract No. 35, EMBO, Heidelberg
2. Surowy, C.S. & Whish, W.J.D. (1980) Biochem. Soc. Transac. 8, 174-175
3. Purnell, M.R., Stone, P.R., Surowy, C.S. & Whish, W.J.D. (1980) Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, 285-293, Elsevier North-Holland, Inc. (Smulson, M.E. & Sugimura, T., eds.)
4. Purnell, M.R., Stone, P.R., Surowy, C.S. & Whish, W.J.D. (1981) Biochem. Soc. Transac. In the press

A P P E N D I X

General Procedure for T.l.c. Analyses

In most cases samples (approx. 1,500 - 3,000 c.p.m.) were applied directly to the t.l.c. plate. However, in several instances it was necessary to modify this procedure. For Figs. 1 and 13, the plate was desalted in anhydrous methanol, after application of sample, as described in Section 2.2.13; for Figs. 3, 4 and 5, samples were neutralized with 1M HAc before application; for Figs. 11 and 12, samples were lyophilized, redissolved in distilled H₂O and half the volume applied.

Fig. 1 T.l.c. Analysis of Snake Venom Phosphodiesterase Digest Using 1M HAC/O.9M HAC-O.3M LiCl

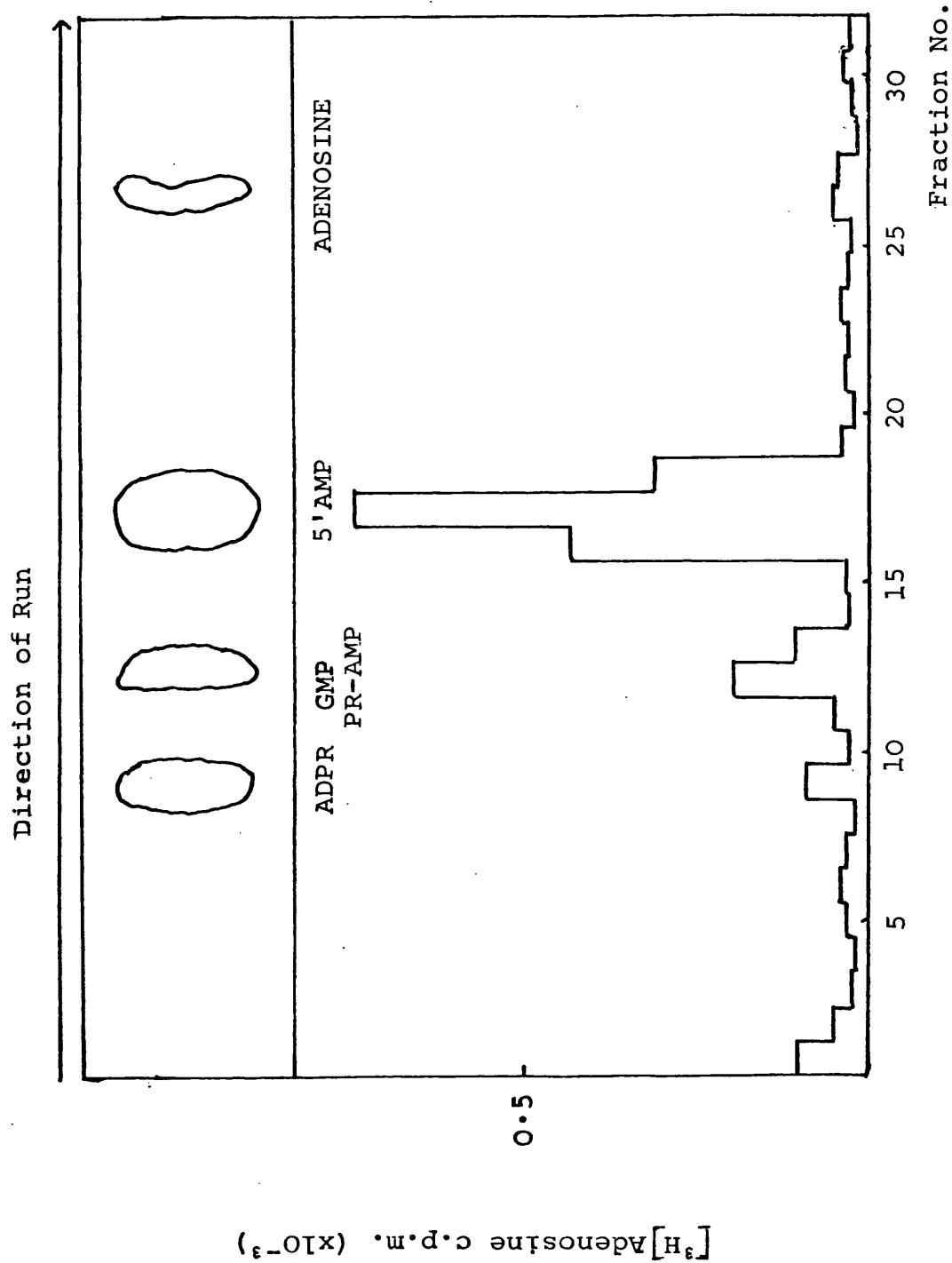


Fig. 2 T.l.c. Analysis of Alkaline Phosphatase Digest Using Double-Distilled Water

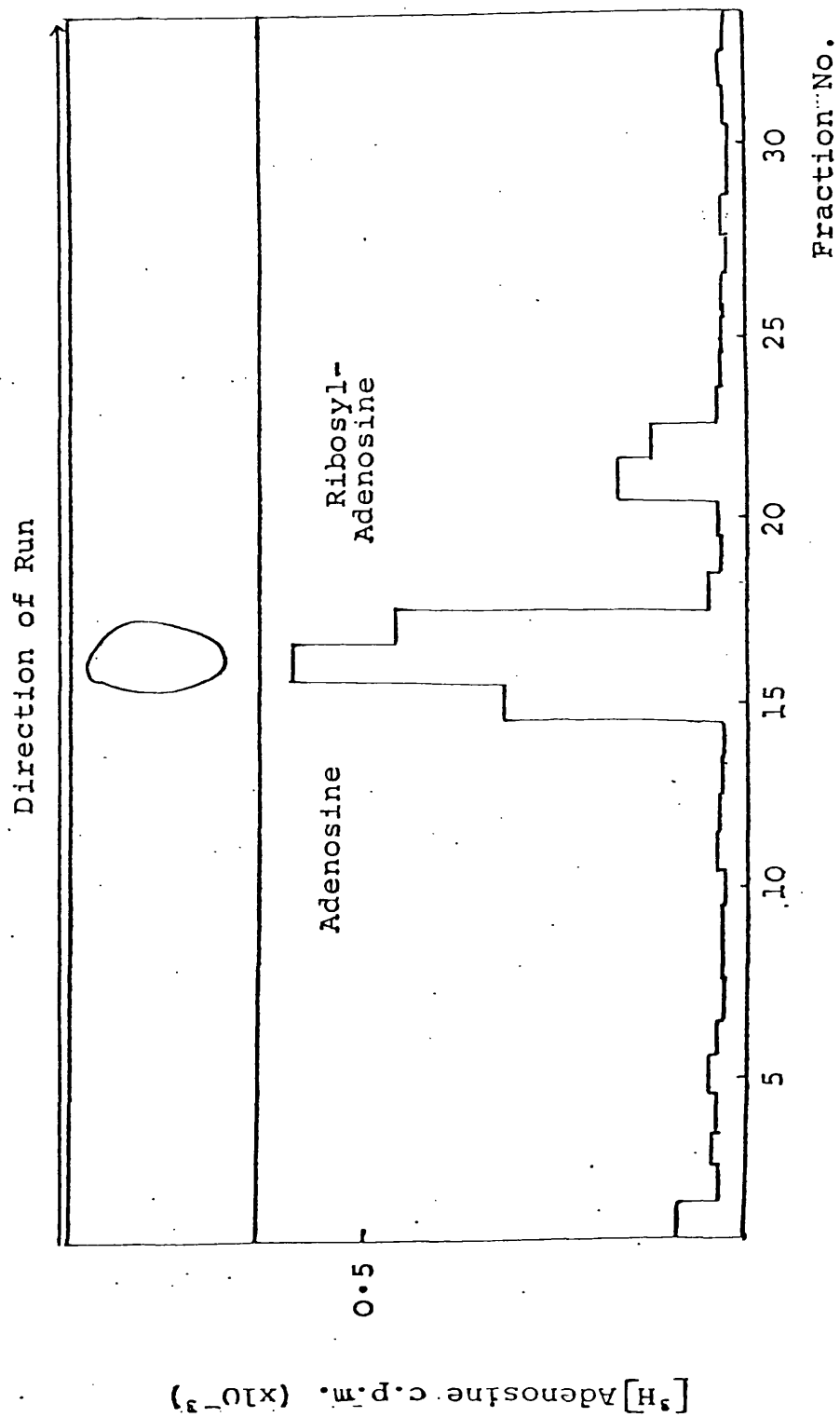


Fig 3. T.l.c. Analysis of Base Digest Using LiCl/EtOH/
 $(\text{NH}_4)_2\text{BO}_3$

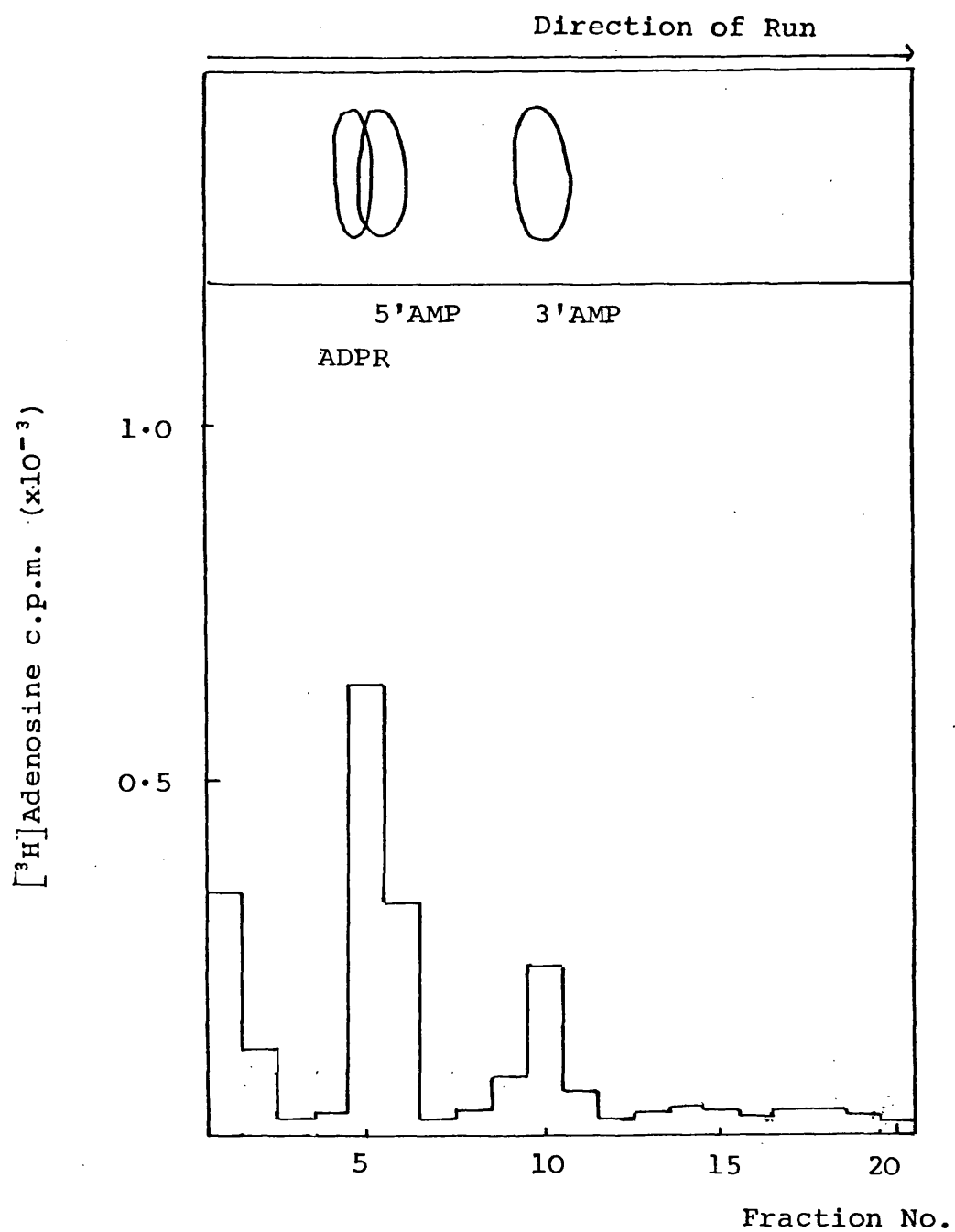


Fig.4 T.l.c. Analysis of Base Hydrolysis Using 1M HAc then
0.3M LiCl

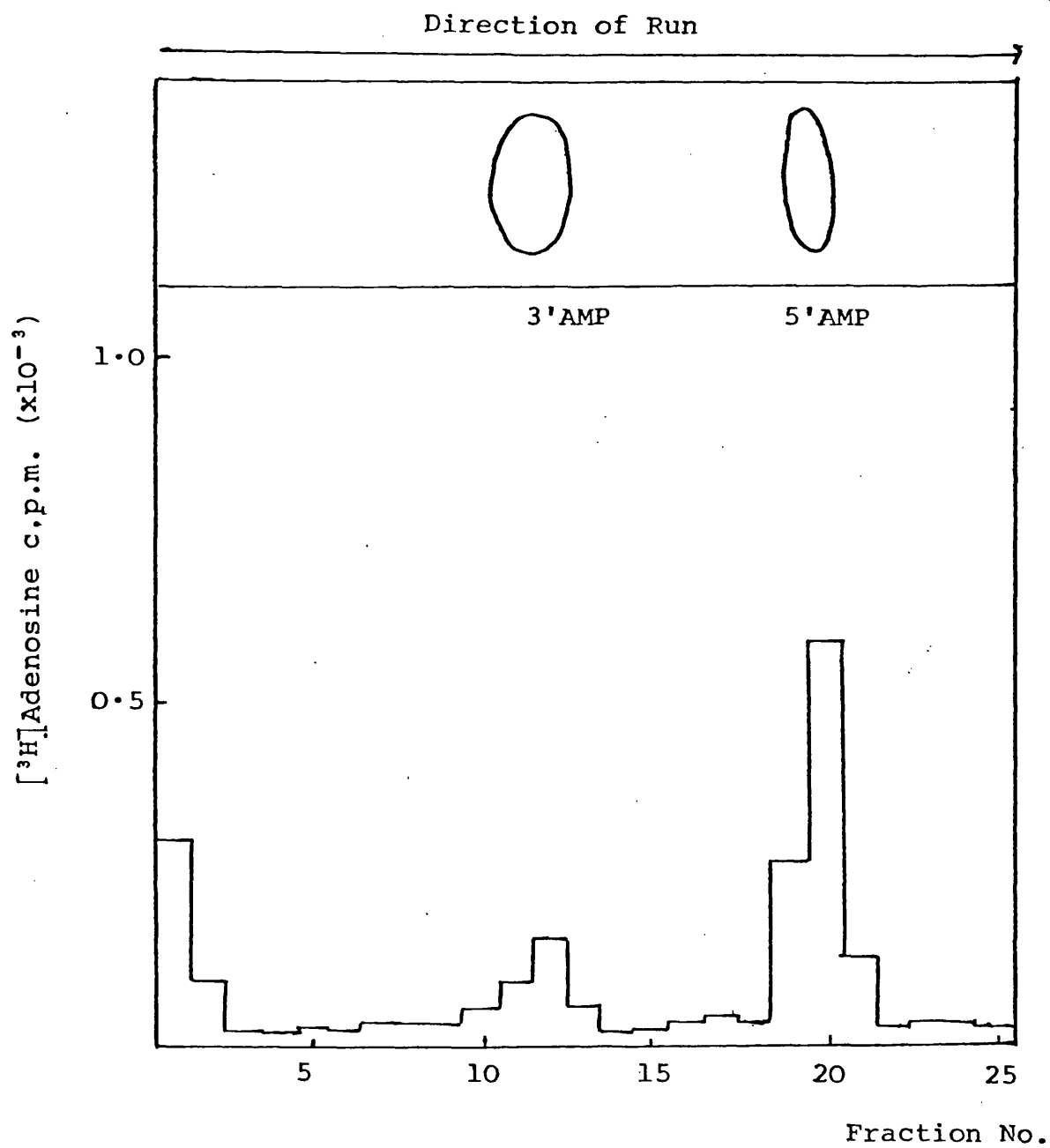


Fig. 5 T.l.c. Analysis of Base Digest Using Sodium Tetraborate and Boric Acid

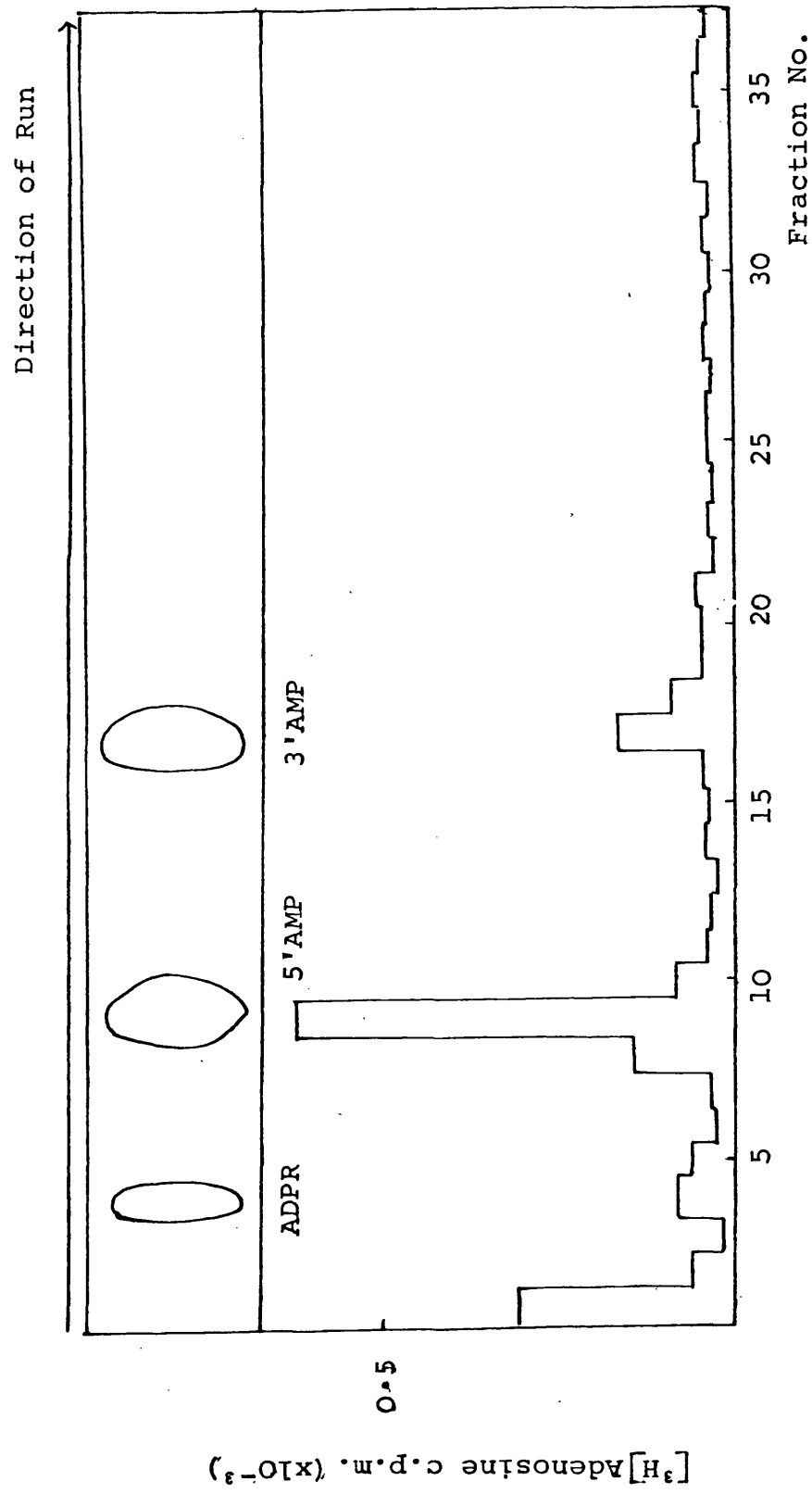


Fig.6 T.l.c. Analysis of 5' Nucleotidase Digest of Base Hydrolysis Using 1M HAC/
0.9M HAC - 0.3M LiCl

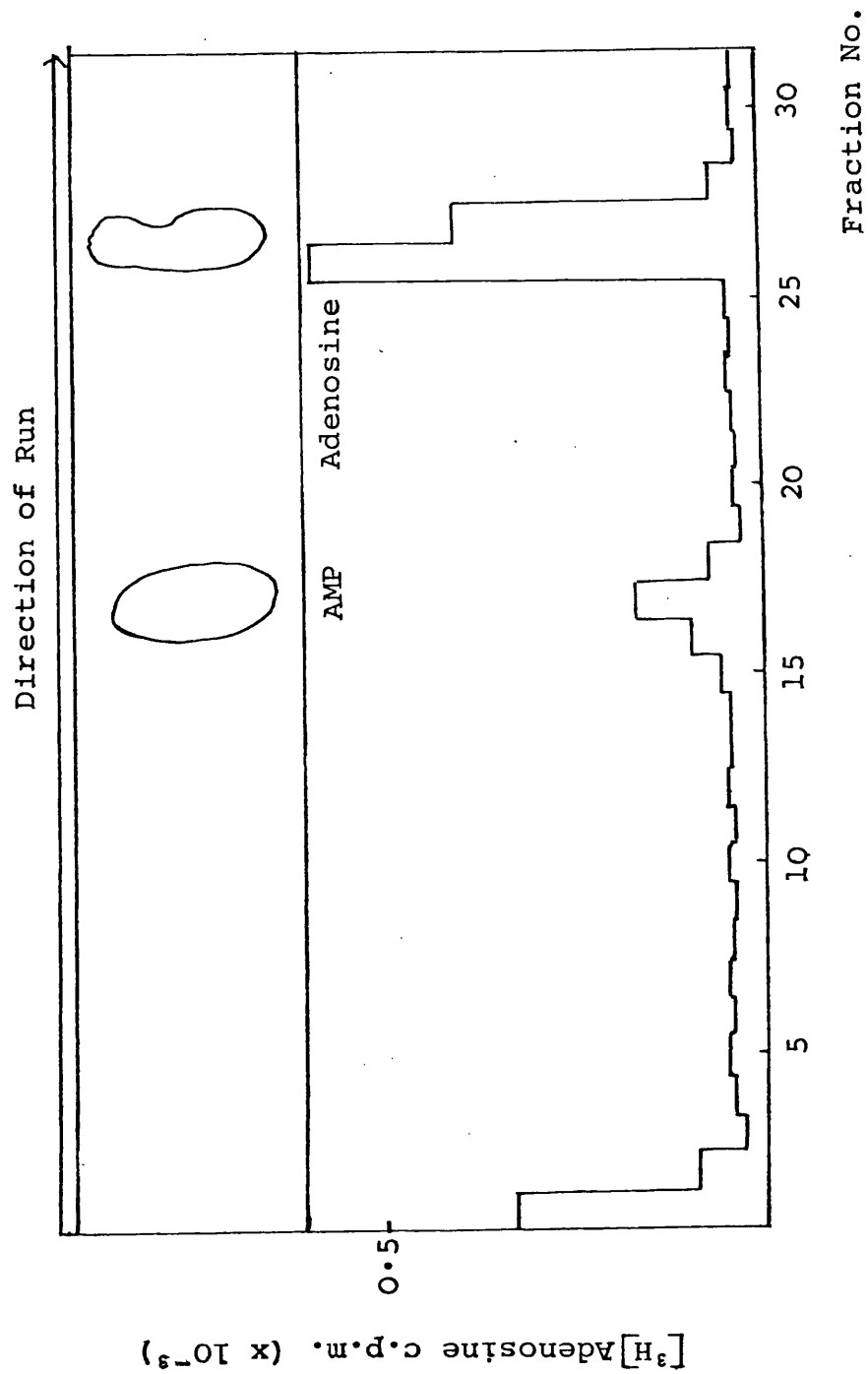


Fig. 7 T.l.c. Analysis of 3' Nucleotidase Digest of Base Hydrolysis Using
 1M HAC/0.9M HAC - 0.3M LiCl

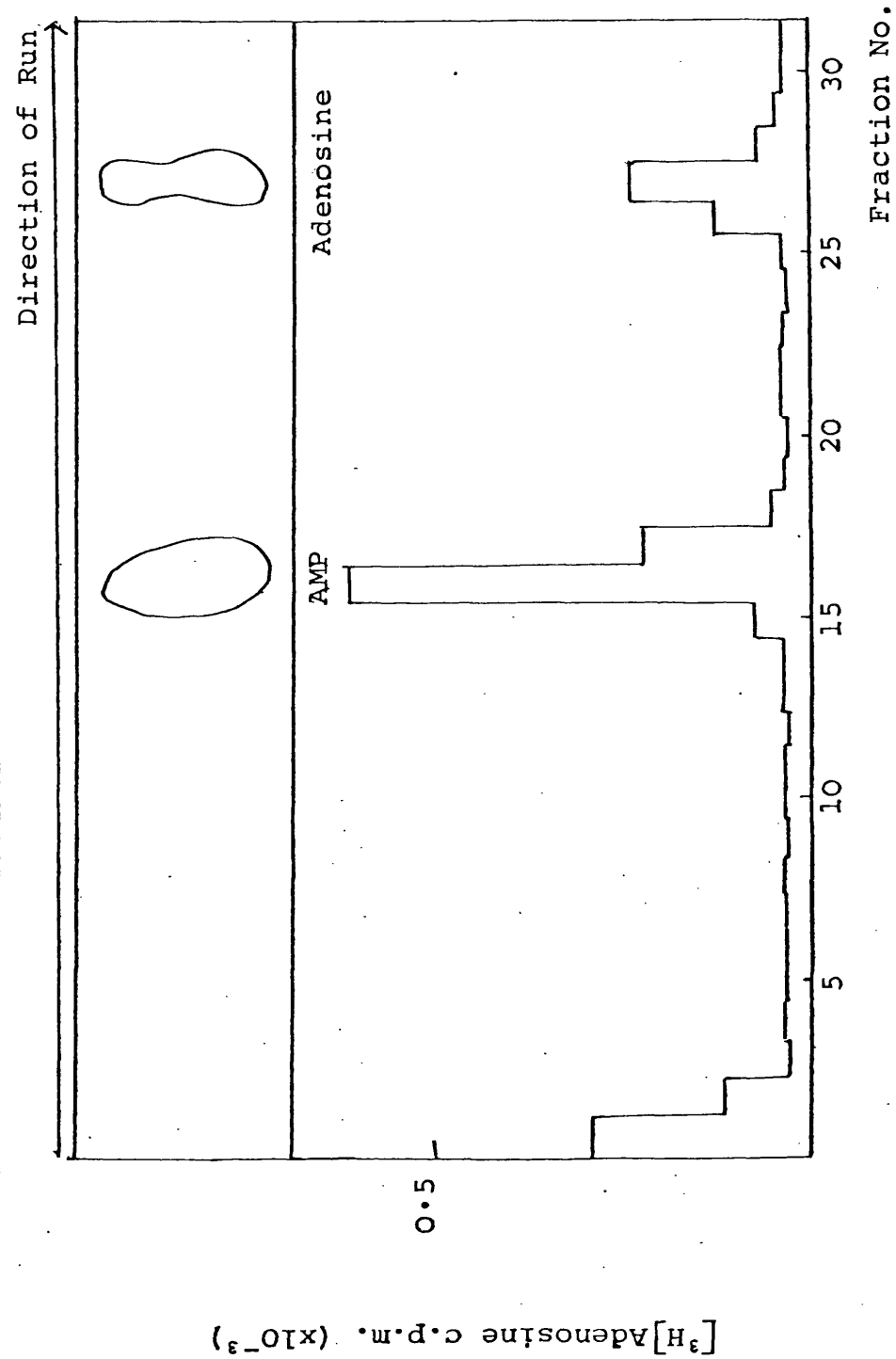


Fig. 8 T.l.c. Analysis of Hydroxylamine Hydrolysis Using 1M HAC/0.9M HAC - 0.3M LiCl

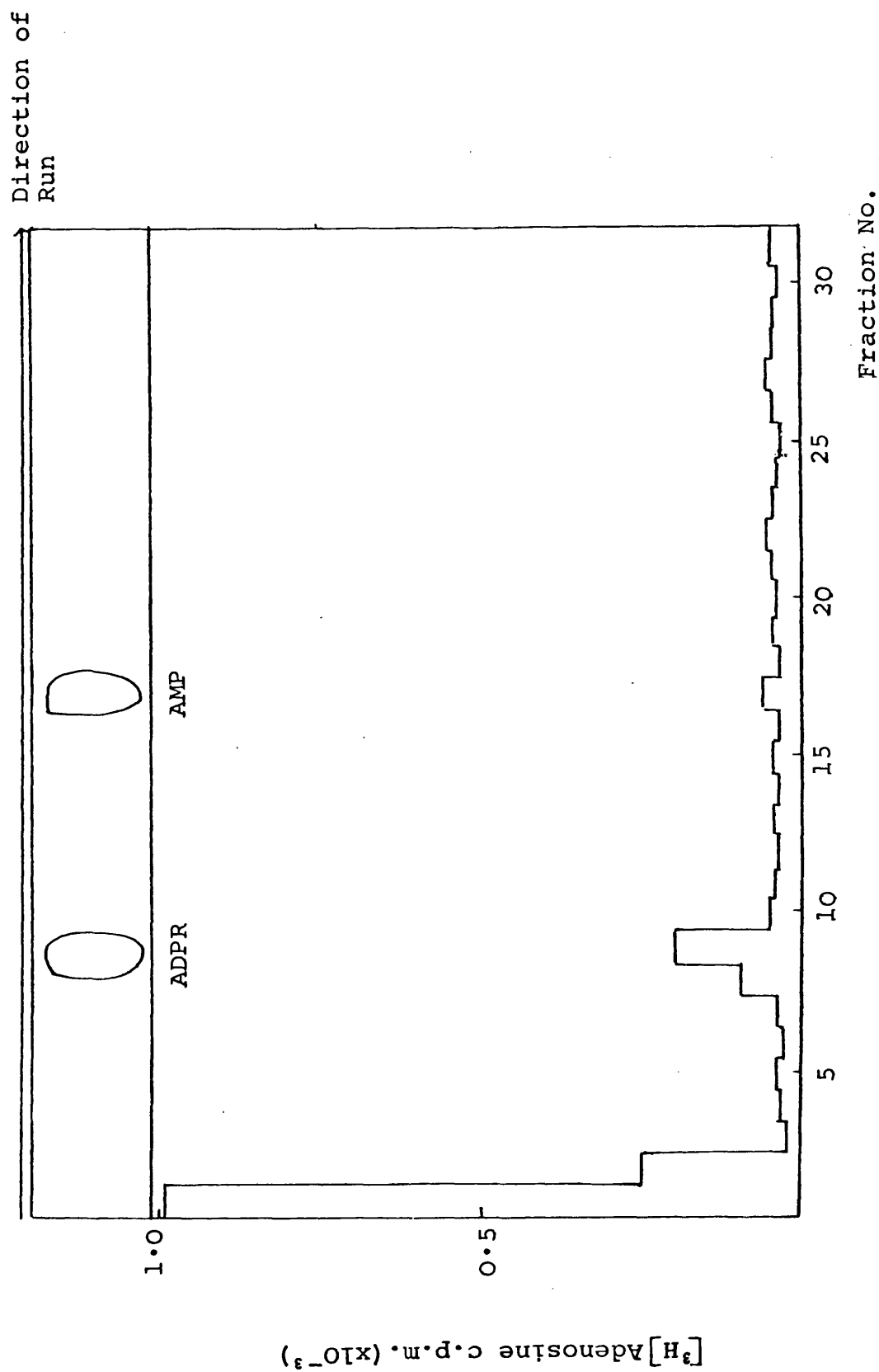


Fig. 9 T.l.c. Analysis of Glycohydrolase Digest Using 1M HAC/0.9M HAC - 0.3M LiCl

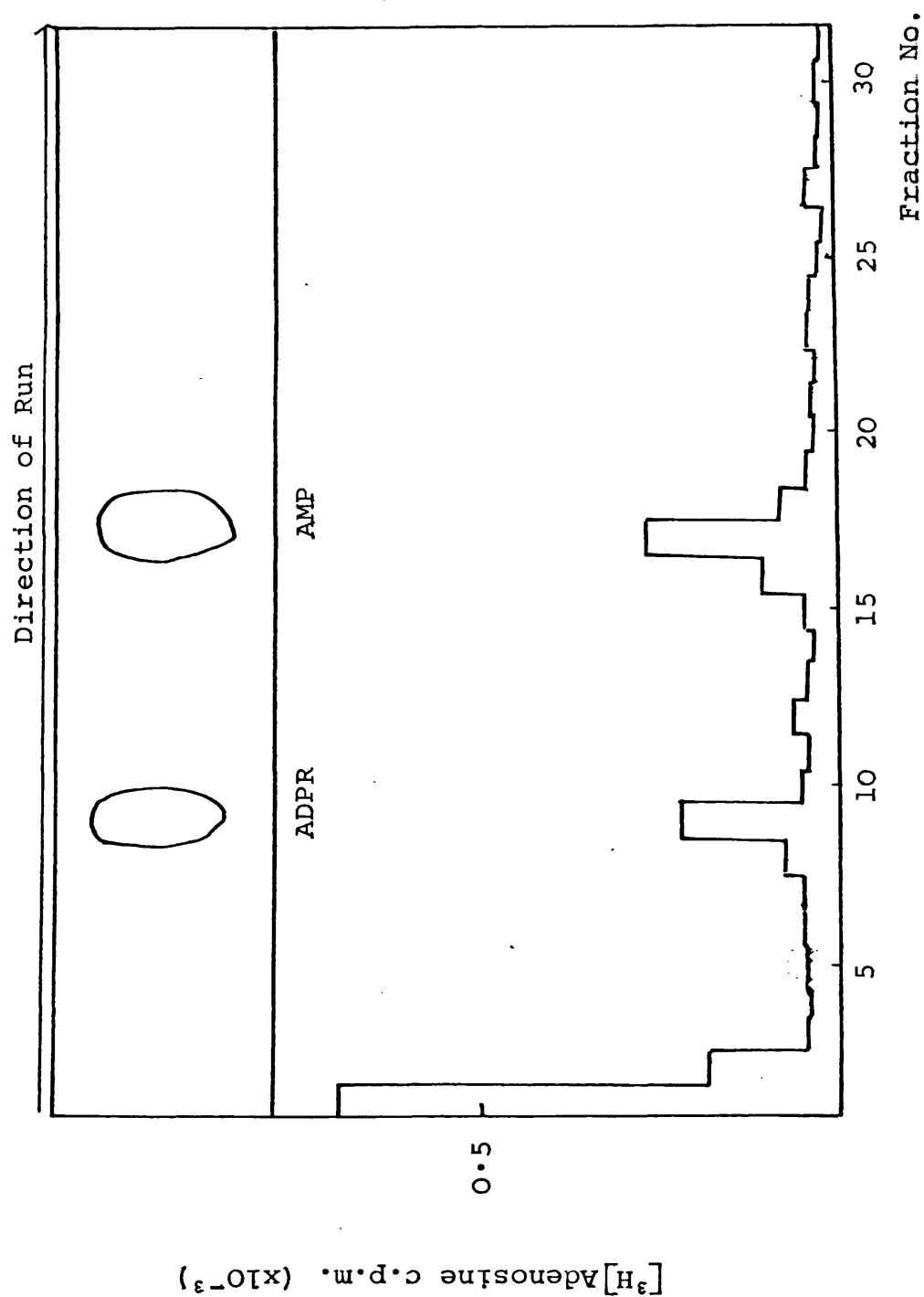


Fig. 10 T.l.c. Analysis of Deoxyribonuclease/Snake Venom
Phosphodiesterase/Alkaline Phosphate Digest Using
LiCl/EtOH/(NH₄)₂BO₃ System

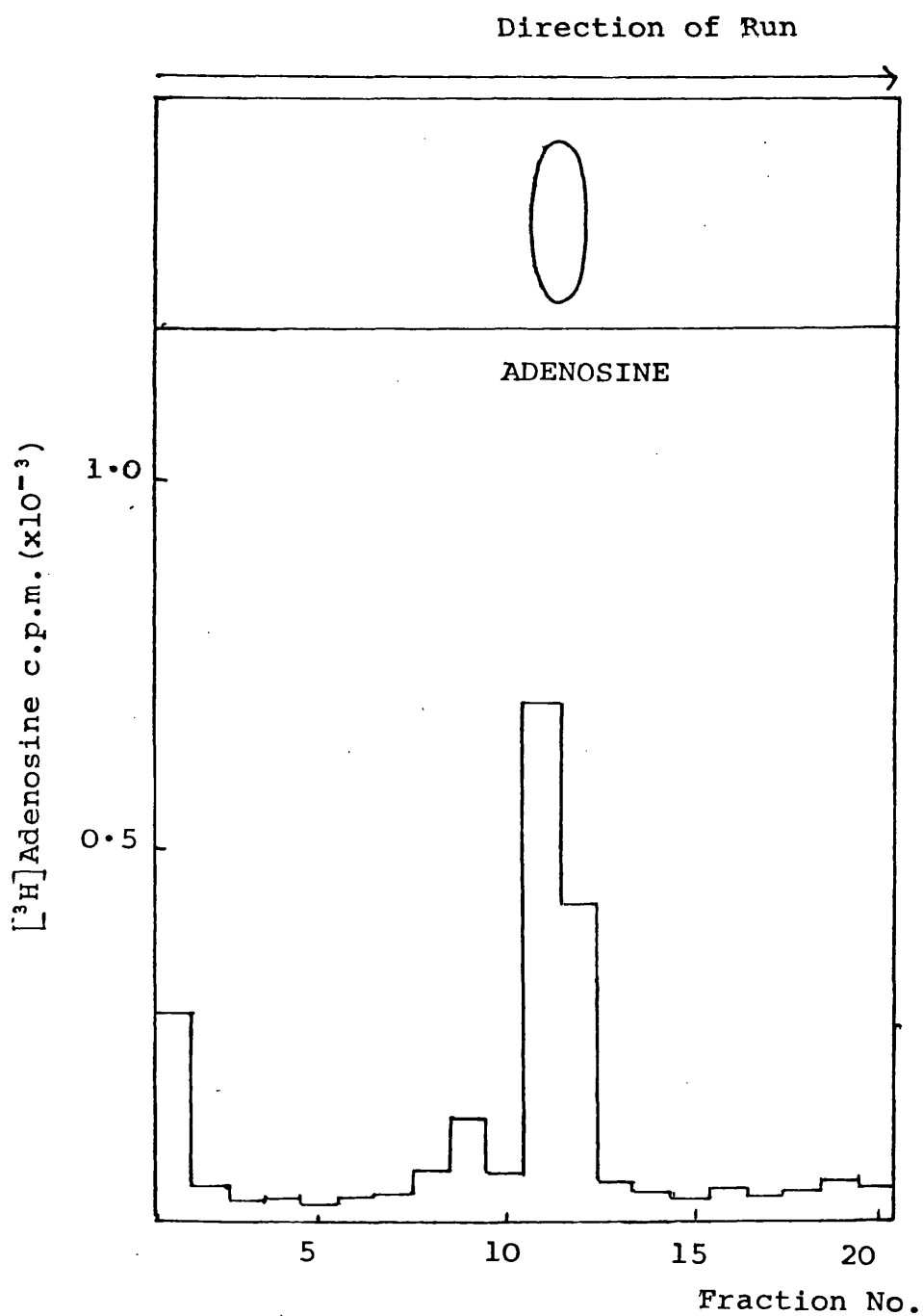


Fig. 11 T.l.c. of 6M Acetic Acid Fraction from Aminoethyl-Cellulose Chromatography
Using Sodium Tetraborate/Boric Acid

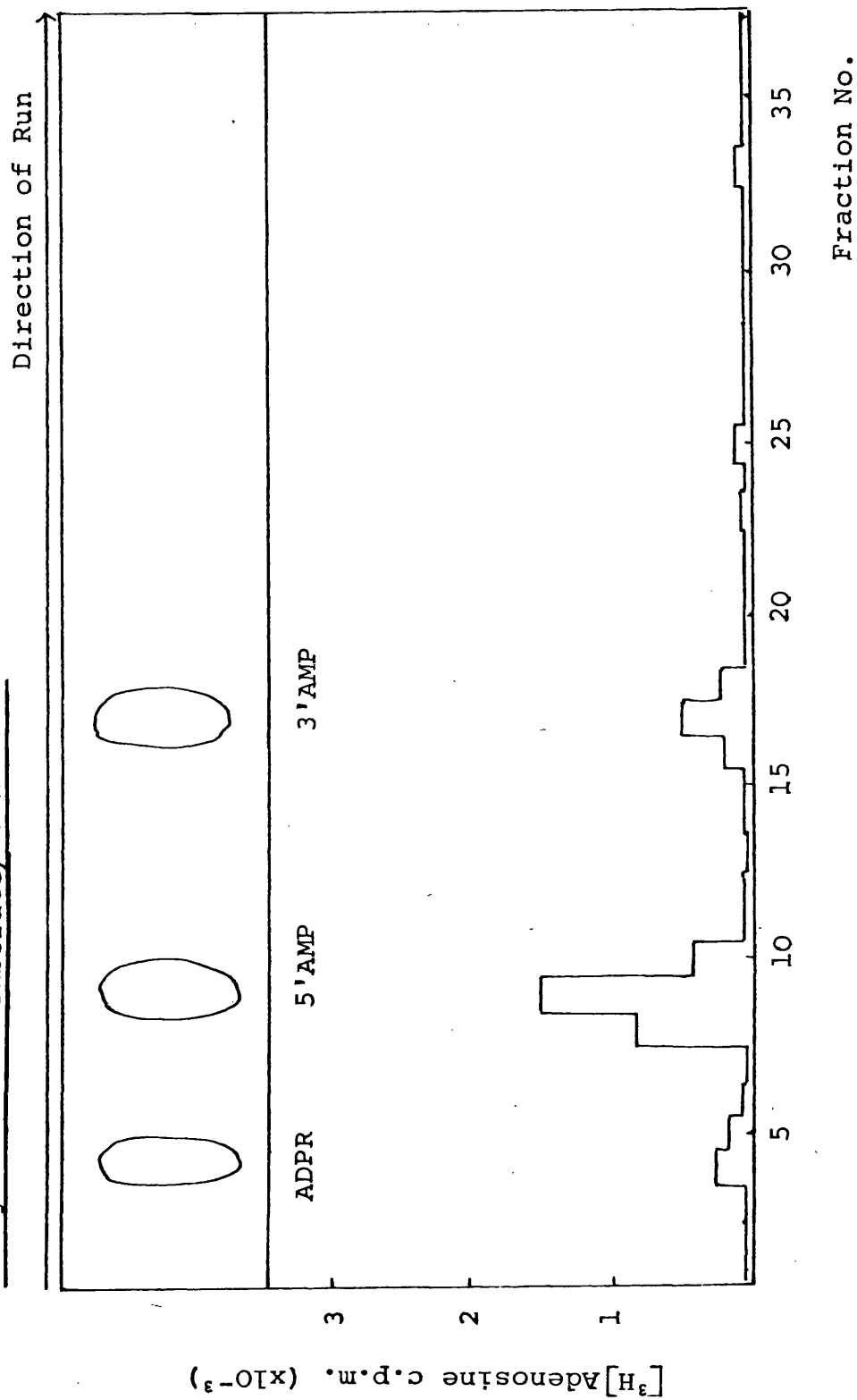


Fig. 12 T.l.c. of 1M Ethylamine Fraction from Aminoethyl-Cellulose Chromatography Using Sodium Tetraborate/Boric Acid

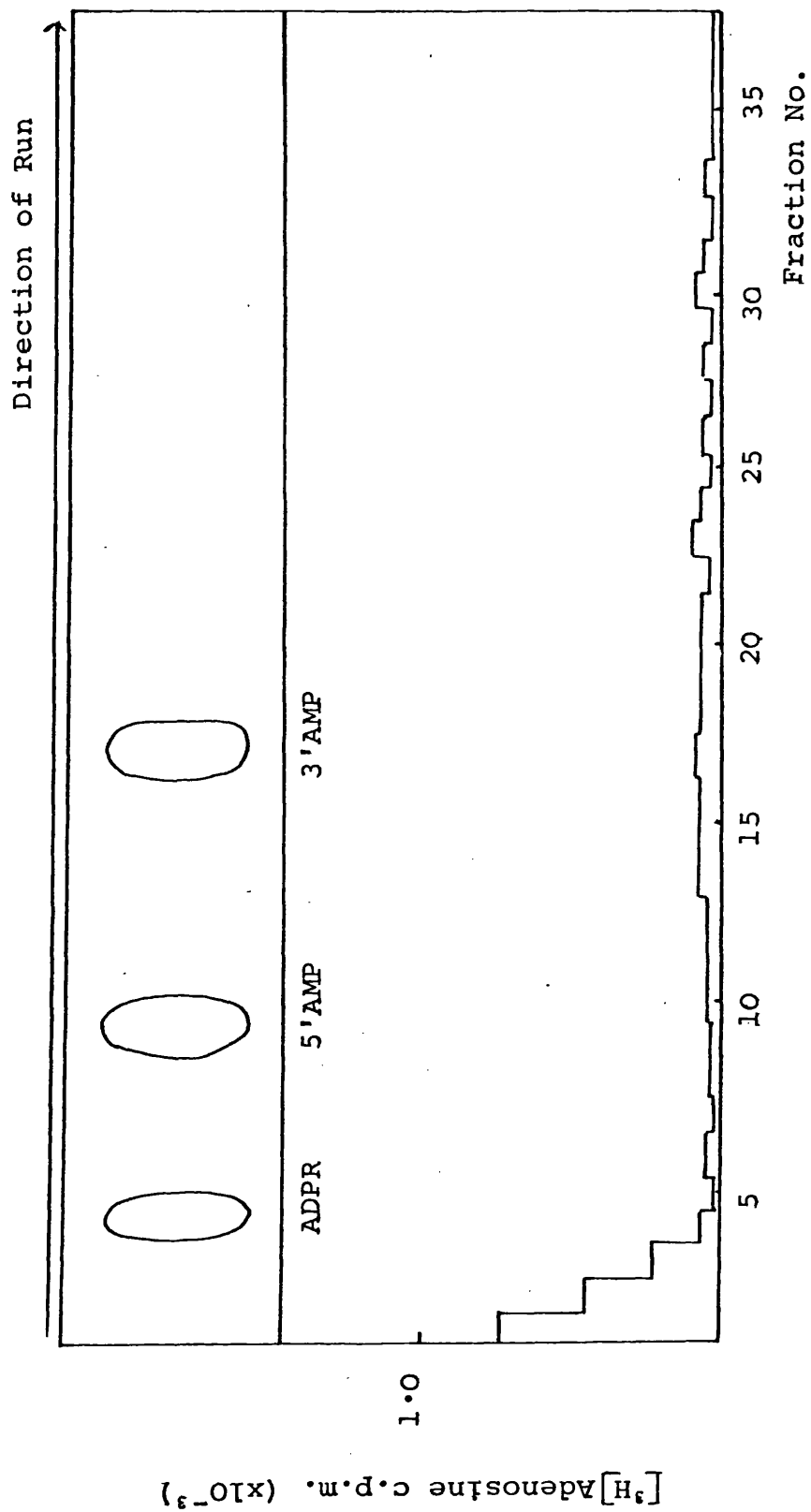


Fig. 13 T.l.c. Analysis of Snake Venom Phosphodiesterase Digests of Ethylamine Fraction
Using 1M HAC/0.9M HAC - 0.3M LiCl

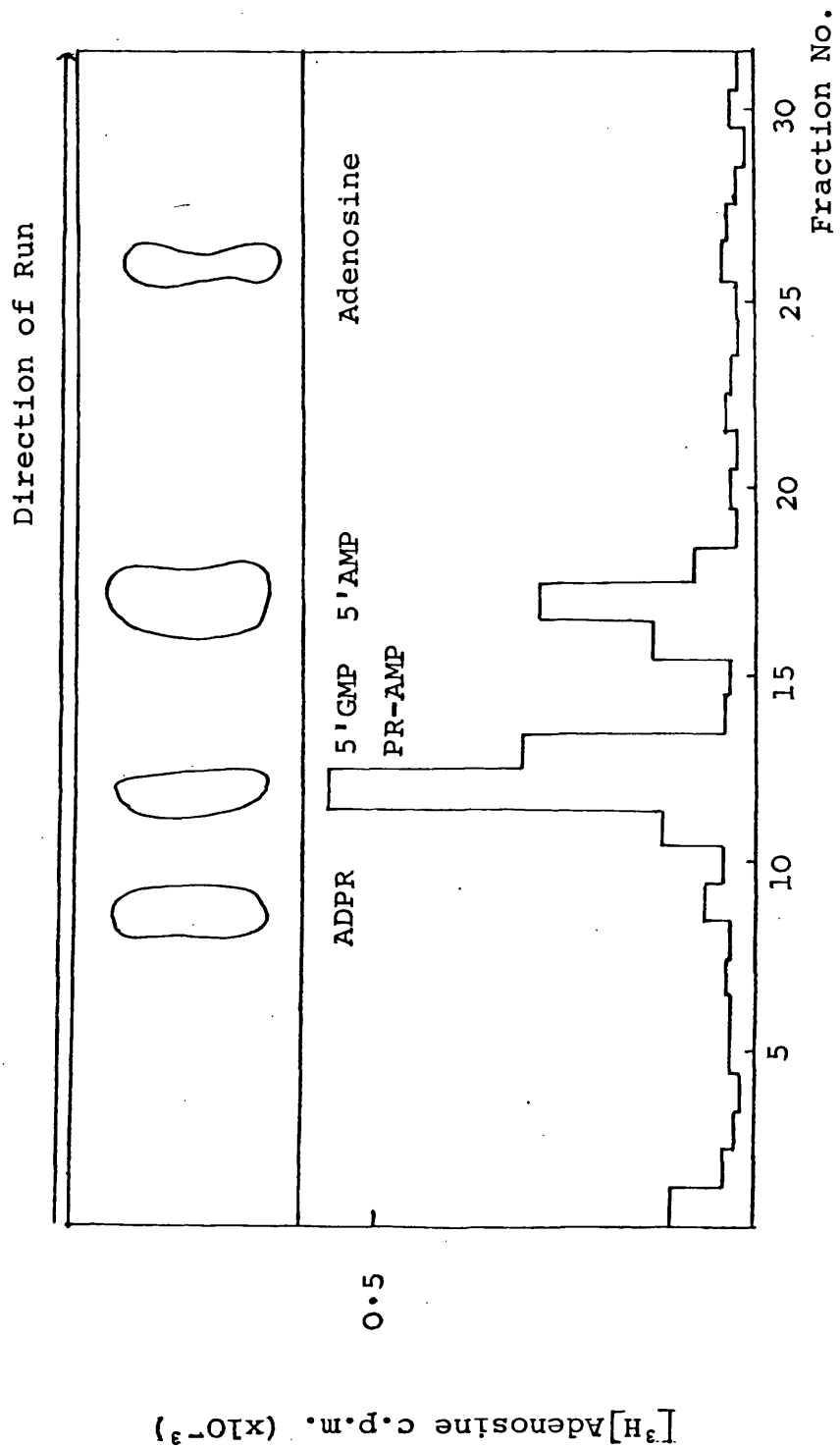


Fig. 14A HAc/HAc-LiCl T.l.c. of Snake Venom Phosphodiesterase Digest of Mouse L1210 Cells
Incubated with 3-Aminobenzamide

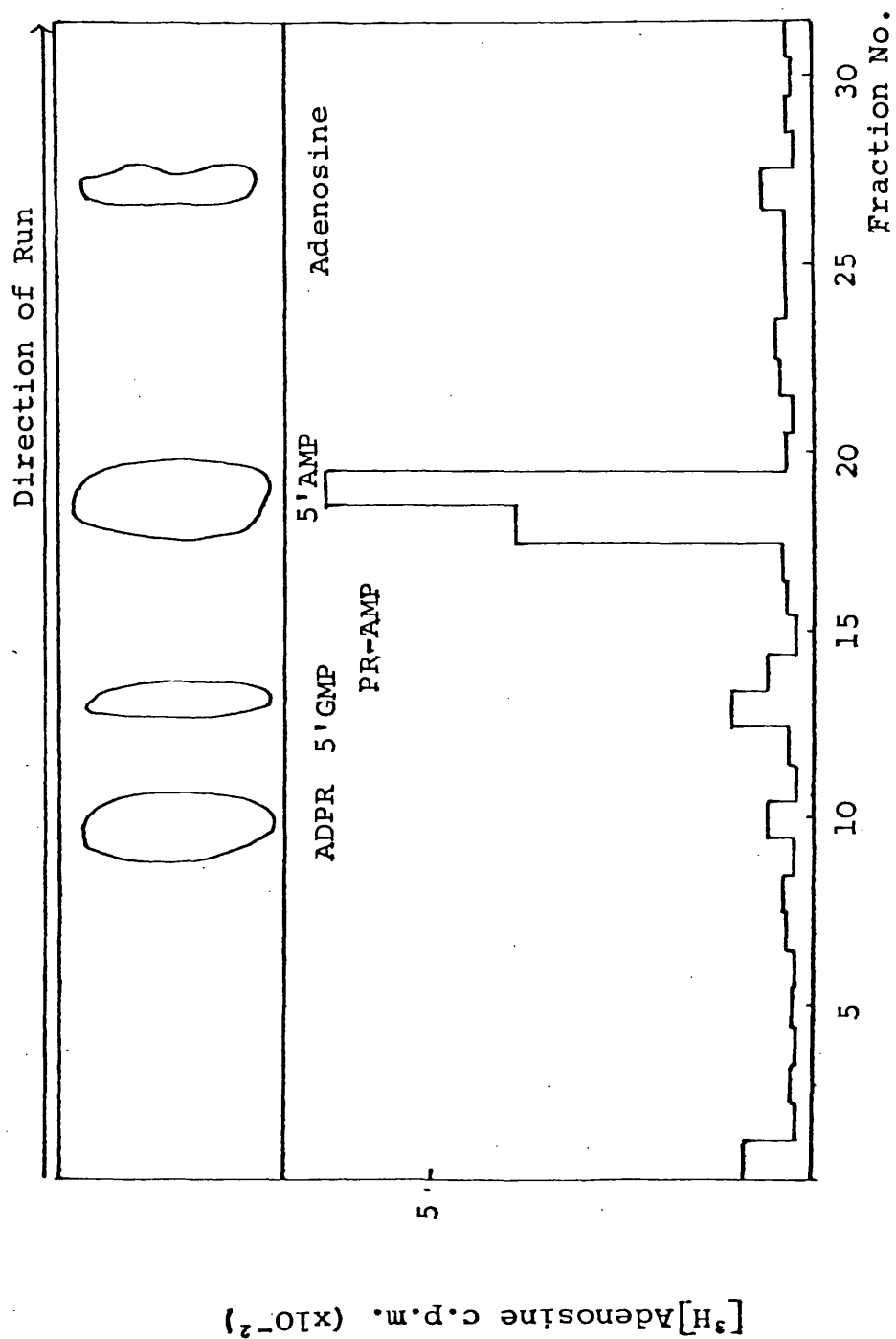


Fig. 14B HAC/HAC-LiCl T.l.c. of Snake Venom Phosphodiesterase Digest of Mouse L1210 Cells
Incubated with Thymidine

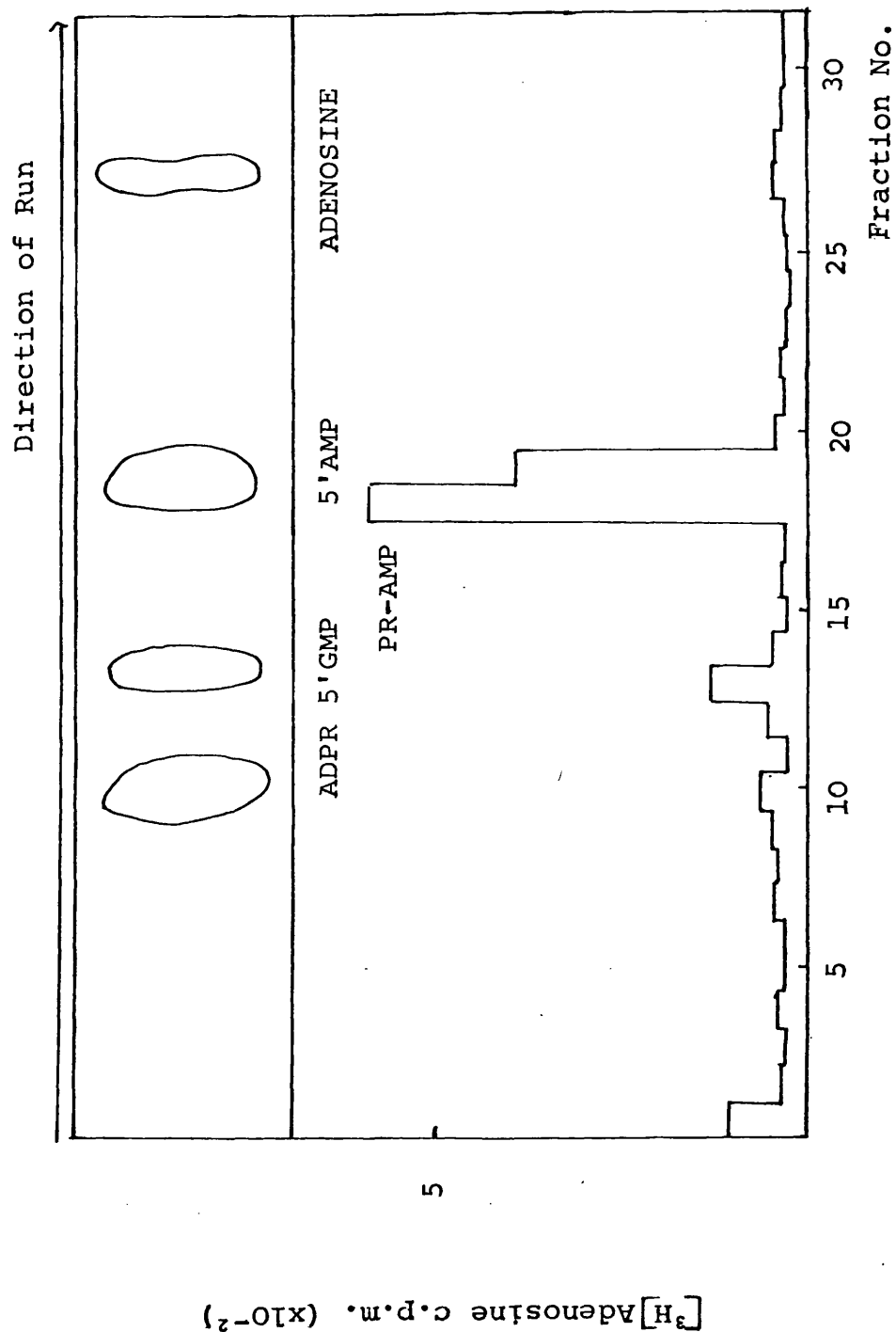


Fig. 15A T.l.c. Analysis of Base Hydrolysis of 3-AB-Treated Cells.

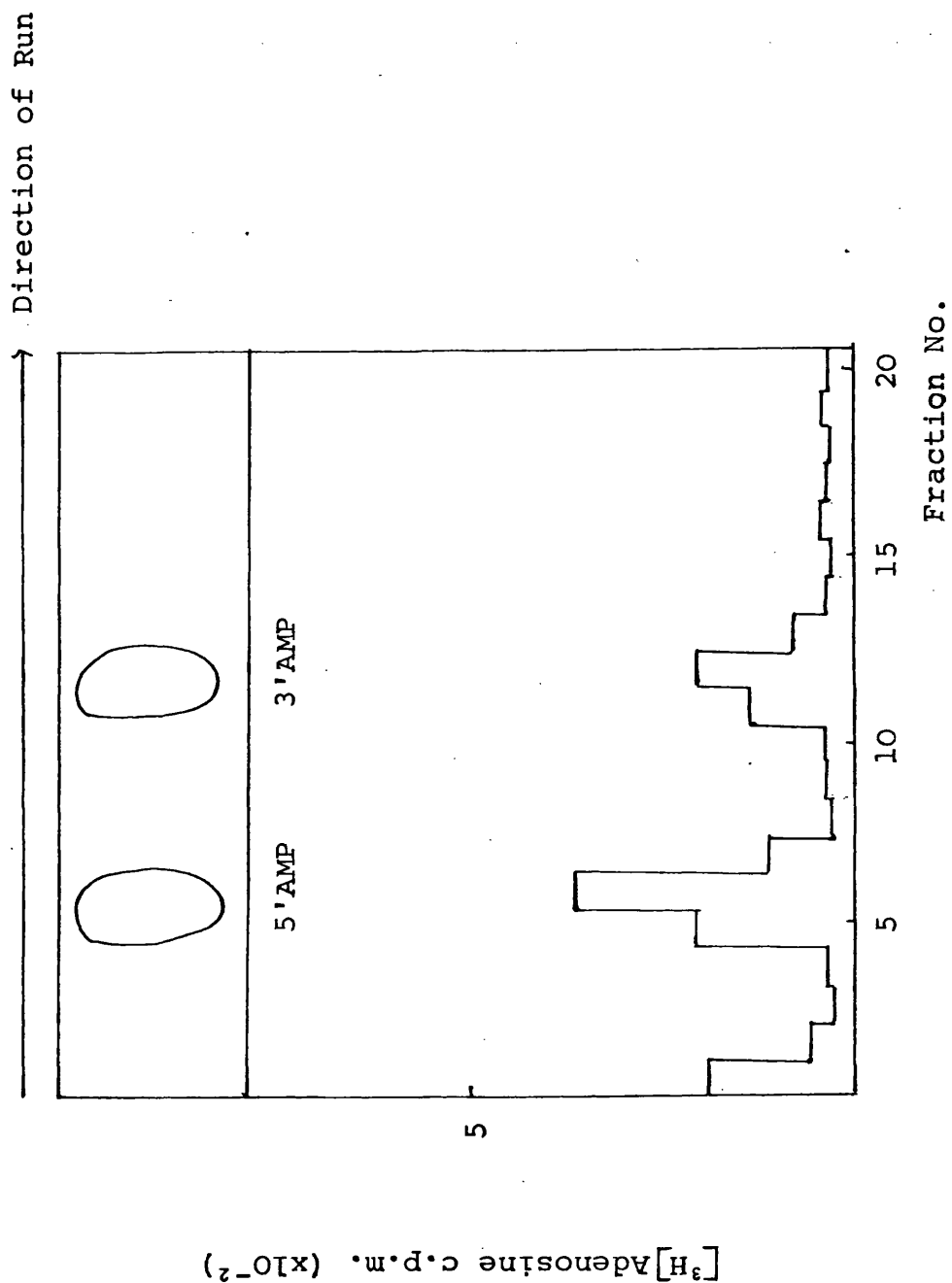


Fig. 15B T.l.c. Analysis of Base Hydrolysis of Thymidine-Treated Cells

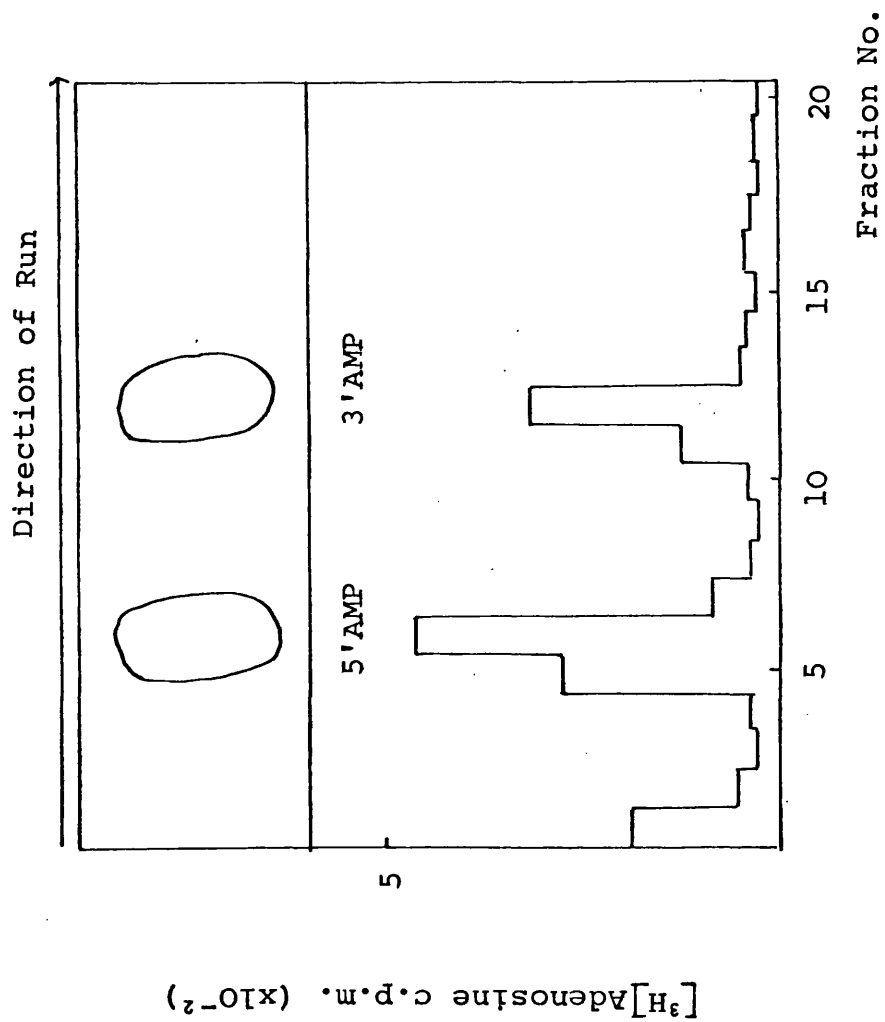


Fig. 16A T.l.c. Analysis of Acetic Acid Fraction of Aminoethyl Cellulose Chromatography of 3-Aminobenzamide-Treated Cells

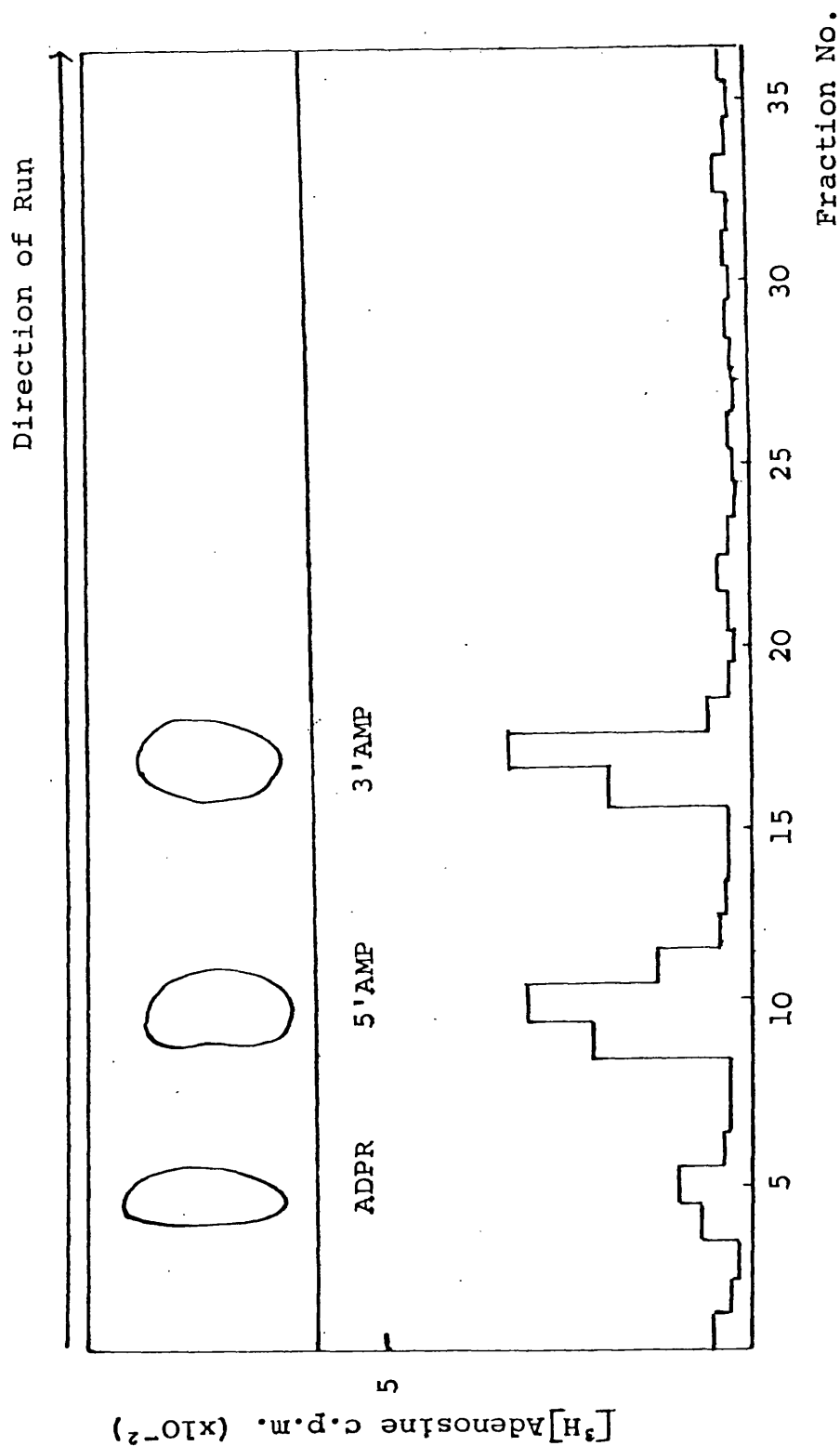


Fig. 16B T.l.c. Analysis of Acetic Acid Fraction of Aminoethyl Cellulose Chromatography
 of Thymidine-Treated Cells

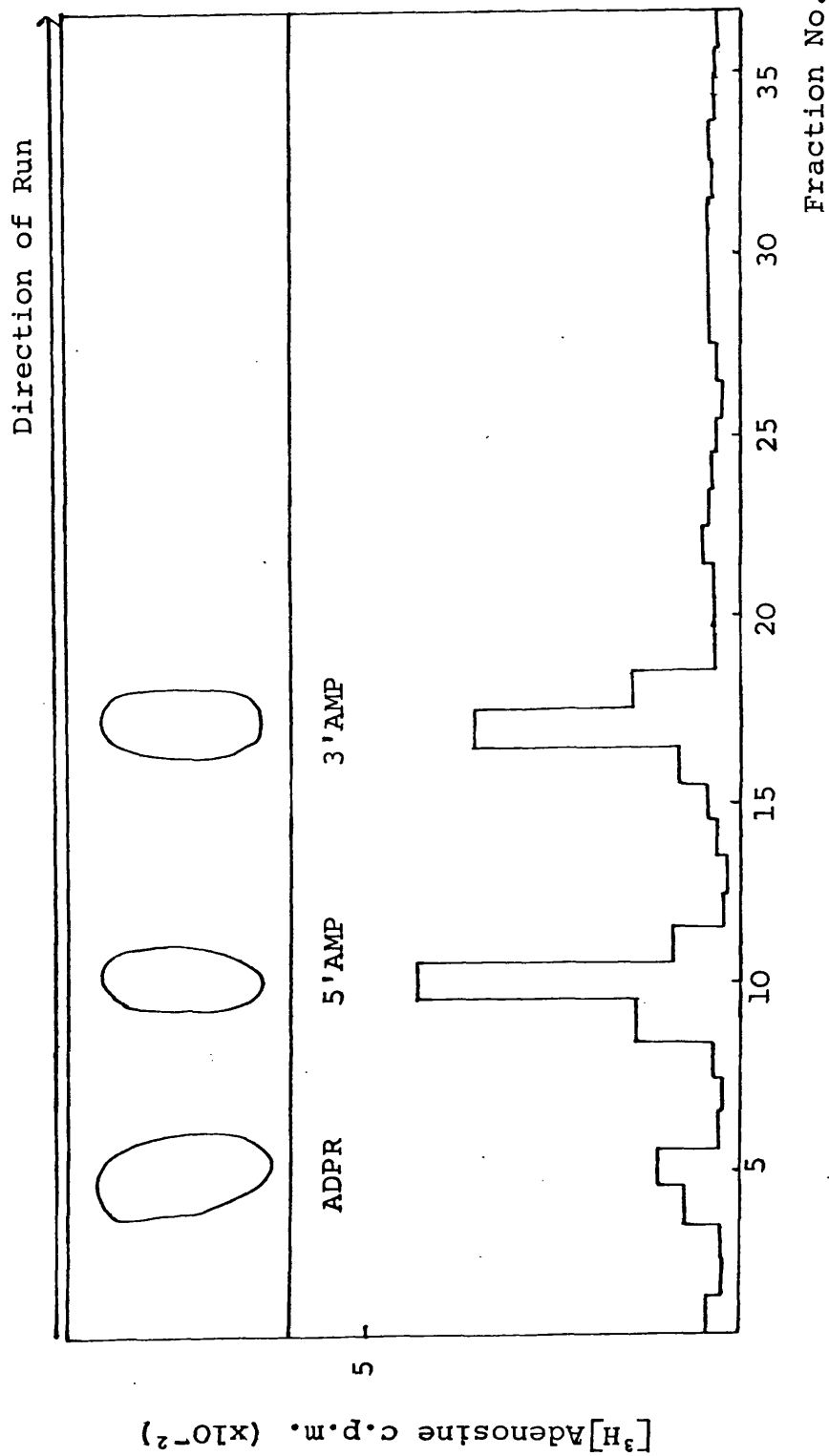


Fig. 17A T.l.c. Analysis of Snake Venom Phosphodiesterase Digest of Ethylamine Fraction of Aminoethyl Cellulose Chromatography of Base-Treated Sample Pre-Incubated with 3-Aminobenzamide

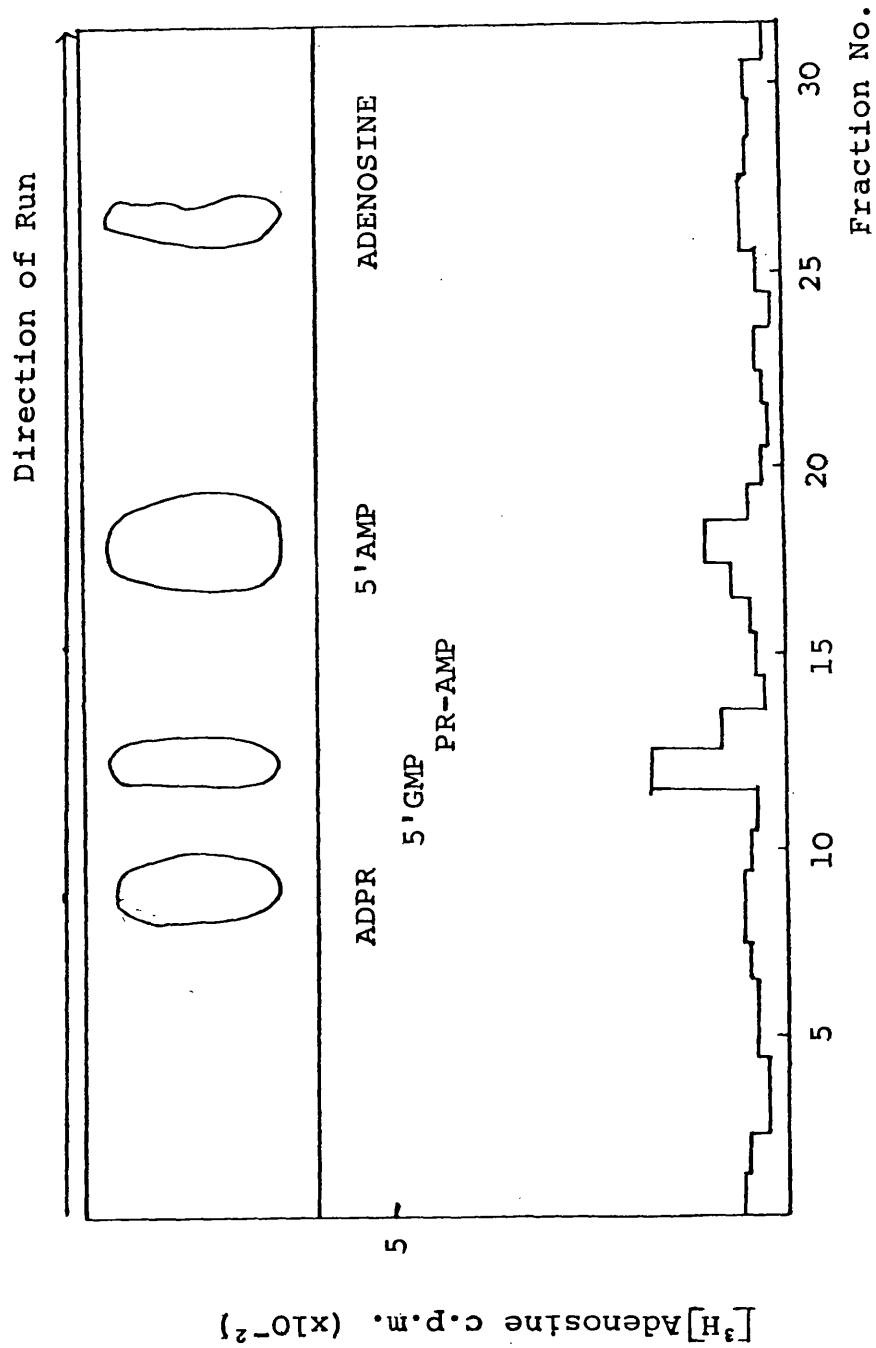


Fig. 17B T.l.c. Analysis of Snake Venom Phosphodiesterase Digest of Ethylamine Fraction of Aminoethyl Cellulose Chromatography of Base-Treated Sample Pre-Incubated with Thymidine

